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14. ABSTRACT
Shock induces loss of energy dependent volume control mechanisms, which cause cell and tissue swelling, compression of capillaries, and impairment of microcirculatory O_2 transfer. The objective of the study was to increase the tolerance to the low volume state by passively moving water from the cell back into the microcirculation by loading patients with specialized cell impermeant agents. This decompresses and reloads the capillaries, and increases the efficiency of low volume oxygen transfer. Rats or Pigs were hemorrhaged in lactate controlled models and given various impermeant based low volume resuscitated (LVR) solutions. Outcomes were determined by cardiovascular, metabolic, and tolerance endpoints. Small cell impermeants and PEG-20k (hybrid impermeant) in LVR solutions, relative to saline and Hextend controls, increased tolerance to the low flow state by 2 to >16 fold, normalized arterial pressure, lactate, and tissue swelling during LVR, caused rapid O_2 debt repayment, increased regional capillary flows, expanded intravascular volume (autotransfusion), and caused survival (100% Vs 0%). PEG-20k uniquely possessed both impermeant and colloidal properties (hybrid), which produced potentiating effects. Conclusions: Ischemia-induced cell and tissue swelling is a key mediator of resuscitation injury, which can be mitigated by stable cell impermeants in LVR solutions. This dramatically increases the tolerance to the low volume state and survival.

15. SUBJECT TERMS
Cell Impermeants, gluconate, ischemia, resuscitation, low volume resuscitation, PEG-20k

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INTRODUCTION: The direct and indirect effects of severe and prolonged tissue hypoxia due to hemorrhagic shock are the leading cause of death for battlefield injuries (1, 2). Resuscitation in the field is often seriously inadequate even if the patient makes it to the local field hospital for full resuscitation. The delay to evacuation in far forward units could take many hours to days so time becomes critical and maintaining patients in a low volume status for long periods is a real possibility (3). Work done in this proposal will significantly increase this safe time period. Past resuscitative attempts have focused on maintaining tissue perfusion and oxygen delivery to rebalance the oxygen supply-to-demand ratio by administration of large volumes of crystalloid. However, it is now recognized that controlled resuscitation using lower volumes is effective, more economical, and essential for battlefield operations (4). The objective should be to restore as much oxygen delivery in the lowest volume as possible only to patients that will benefit from the therapy. This approach can be improved by using agents in the low volume resuscitation solution that preserve tissue perfusion. One significant impediment to tissue perfusion during low pressure states is no reflow in the microcirculation caused by cell swelling induced by prolonged cellular ischemia. Endothelial cells in the capillaries swell during ischemia and shut off flow through already narrow capillary corridors (5, 6). Parenchymal cell swelling further compromises the microcirculation by compressing the capillary from the outside. The significance of this proposal is that it uses proven and tested technology and solutions developed for organ preservation for transplantation that resolve ischemia-induced cell swelling and applies it to shock and resuscitation injury. Specifically, the use of simple cell impermeants in low volume resuscitation solutions will significantly attenuate cell swelling, capillary no reflow, and preserve DVO_2 to critical tissues both before and after full resuscitation. This lessens end organ failure and improves survival since distribution of the limited oxygen availability is enhanced. Additionally, cell swelling per se is lethal to tissues (7, 8) so reversal with impermeants is salutary. A further enhancement of this approach is to combine the use of cell impermeants, which preferentially load into the interstitial space, with an oncotic agent, which stays in the capillary space. This creates a double osmotic gradient to first pull water from the cell (where it has the propensity to go during ischemia) with cell impermeants and then pull water from the interstitial space into the capillary where it belongs, with a colloid like PEG-20k. Thus, attacking one of the root causes of severe resuscitation injury (cell and tissue swelling) using proven methods should have a cascading effect that improves the survival of military personnel from severe hemorrhagic shock and resuscitation injury. These concepts and products will also be useful in civilian treatment of shock. The significance of this approach, beyond the obvious medical benefits to humans, is the simplicity of the concept, its proven track record of use in organ transplantation, and the extreme stability of the active components (cell impermeants). The diagram below (**Figure 1**) summarizes the biological problem and the solution.

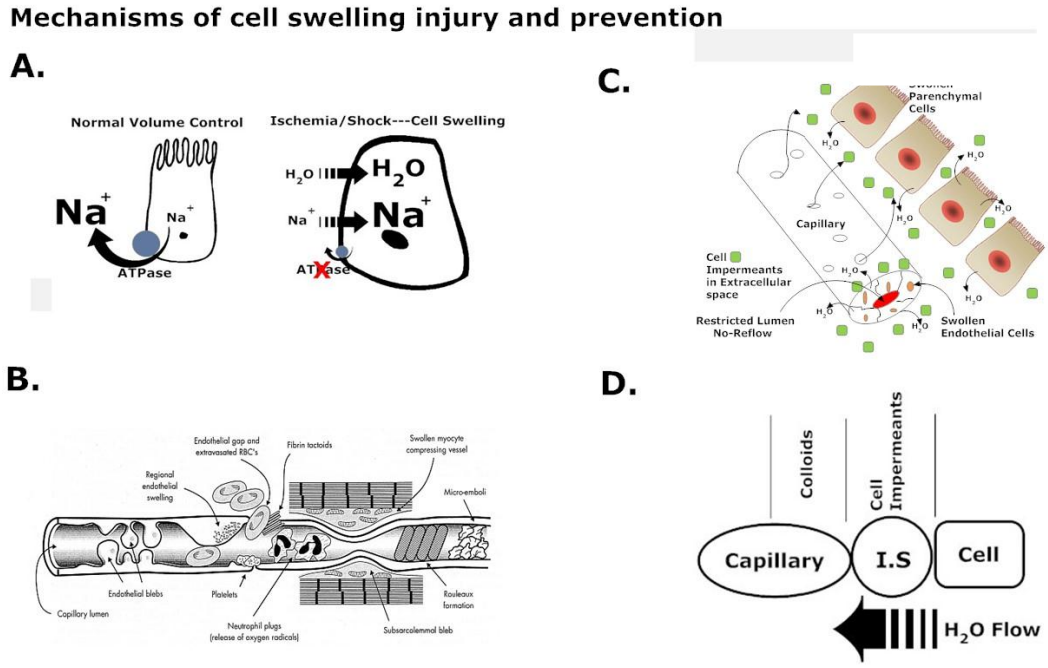


FIG 1. Cells swell as the sodium pump fails from lack of ATP during low flow states. Sodium enters the cell passively and water follows (A). Swollen parenchymal and endothelial cells then impair capillary flow by compression, causing exacerbations in local perfusion during shock (B). Cell impermeants loaded into the interstitial space non-energetically and biophysically prevent water movement into the cell (C) and a dual osmotic gradient is created when cell impermeants are combined with oncotic agents to impel cell water movement out of the cell and into the capillary space where it belongs (D). This reduces cell and tissue swelling and capillary compression AND increases capillary fluid volume, capillary pressure, and microcirculatory flow, which serves to restores microcirculatory oxygen delivery in the low flow state. This increases the tolerance to the low flow state when given in the low volume resuscitation solution and increases the "Golden Hour" on the battlefield. The time that a patient can safely stay in the low volume state until definitive medical care is needed is greatly expanded using this approach.

KEY WORDS: Swelling, Ischemia, Osmotic Forces, Resuscitation Injury, Tolerance, Microcirculation, Perfusion

BODY:

The objectives and specific tasks for the project were;

I. To identify which cell impermeants produced the best effects on tissue swelling
II. To assess these impermeants in LVR solutions in a rodent hemorrhagic shock model
III. To translate the LVR effects to a pre-clinical porcine model of hemorrhagic shock.
IV. To define how this optimized stable crystalloid LVR solutions work (Mechanisms).

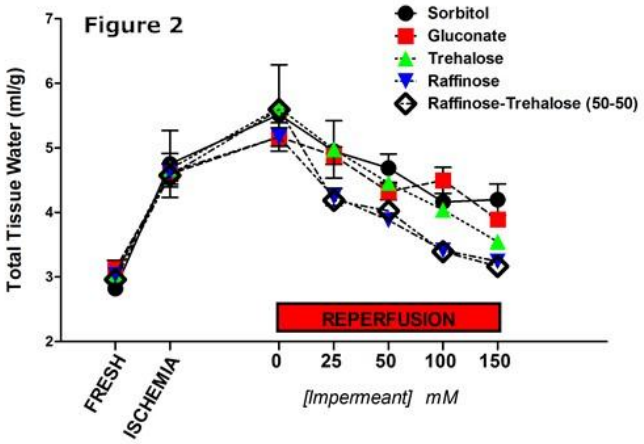
The following experiments and their results were used to address those objectives and tasks.

I. To identify which cell impermeants produced the best effects on tissue swelling:

Cell impermeants are small molecules that are large enough to freely transit across the capillary wall but are too large and / or too charged to enter the cell. Thus, cell impermeants preferentially load into the extracellular space in equilibrium with the capillary space where they serve to pull water out of the cell. More accurately, they prevent water from moving into the cell secondary to ionic shifts in the cell caused by ischemia-induced loss of ATP dependent volume control mechanisms (Na/K ATPase or sodium pump failure). An important attribute of cell impermeant molecules is that they are relatively non-toxic and biologically inert, which is necessary since large amounts are required for biological activity. A concentration of between 60-100 mM in the extracellular fluid compartment is generally needed to prevent cell swelling secondary to ischemia (9). The molecules tested were:

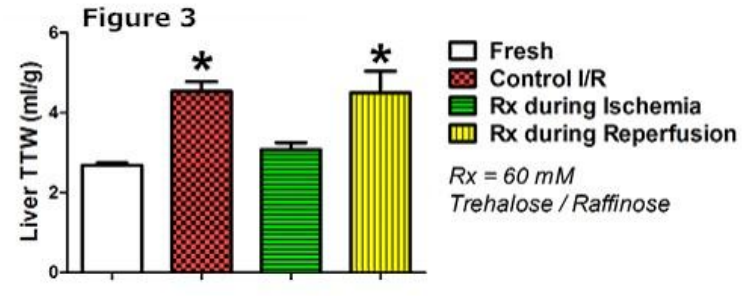
- Sorbitol
- Gluconate
- Trehalose
- Raffinose

These agents have been extensively used in organ preservation solutions so their safety and efficacy in vital organs is well known and established (5-8). In this experiment, mouse livers were excised from anesthetized mice. Slices of liver were prepared in the cold with a Stadie-Riggs microtome to provide a uniform thickness of less than 0.5 mm. Slices were placed in 25-ml Erlenmeyer flasks filled with 1.5 ml of Krebs buffer and incubated for various times in a Dubanoff metabolic incubator at 37° C. Control slices were incubated under an atmosphere of oxygen, while ischemic hypoxia was induced under 95% nitrogen and 5% CO2. Some slices were subjected to 1 hr of ischemia and one hour of oxygenated reperfusion. At various times, some slices were removed from the incubator bath and the wet:dry weight ratios were determined to calculate total tissue water (TTW) and cell swelling. Figure 2 shows the results of these studies when various concentrations of cell impermeants were added during the ischemia time only.



From these data, we conclude that under in-vitro ischemic hypoxia conditions in liver tissue, ischemia-induced cell swelling is reversed by cell impermeants proportional to their extracellular concentration and their molecular weight.

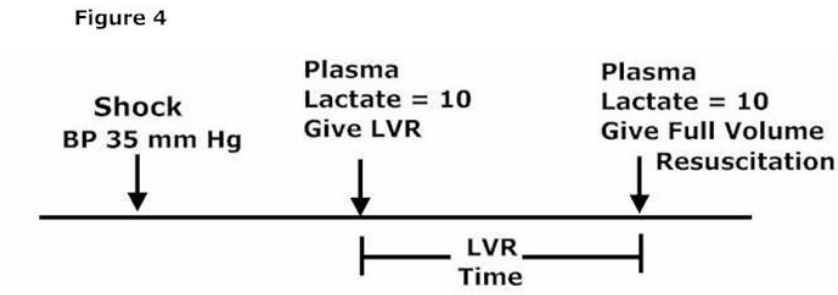
Next, we explored the timing of cell impermeants during ischemia. Specifically, we explored if cell impermeants are active during the ischemia period or during the reperfusion period. This is vital to know if they are to be transitioned to clinical use in battlefield shock since the timing dictates when they can effectively be used. Figure 3 shows the timing dependency of cell impermeants in the tissue slice model.



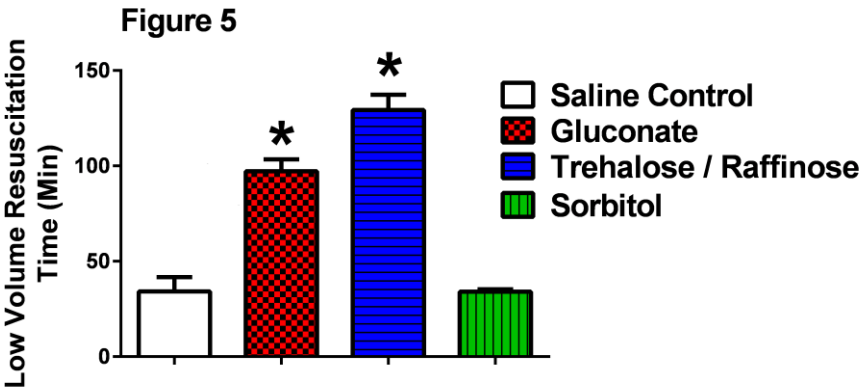
Clearly, the most effective time to deliver cell impermeants to prevent ischemia-induced cell swelling in this model is DURING the ischemia period and not when reperfusion is occurring. Clinically, this means administering cell impermeants during shock and during the low volume state, but not during resuscitation. This fits rather well with the paradigm of battlefield low volume resuscitation where limited amounts of crystalloids are used to help maintain soldiers on the field until evacuation and more definitive resuscitation can occur at a forward field hospital. We see the low volume resuscitation fluid more as a vehicle for drug delivery to shocked soldiers than as volume replacement to increase DVO2. The low volume resuscitation solution used by combat medics will serve as the perfect vehicle to deliver cell impermeants on the field. This will increase capillary perfusion by reducing ischemia-induced cell swelling. The use of these agents in LVR solutions is attractive because they are generally very stable under harsh austere conditions experienced on the battlefield.

II. To assess these impermeants in LVR solutions in a rodent hemorrhagic shock model:

The response of impermeants in the rodent low volume resuscitation model has been well established. Small molecule cell impermeants like gluconate, raffinose, and trehalose significantly increase the tolerance of the shocked patient to the low volume state as indexed by a doubling of the low volume resuscitation (LVR) time. The LVR time is the time from the start of the low volume resuscitation until the start of full resuscitation as shown in the **Figure 4**.



This time represents the "golden Hour" or the time that a shocked victim can safely remain in the low volume state until full resuscitation and definitive medical care is necessary. To test whether cell swelling and the consequences of cell swelling during shock (tissue swelling and microvascular compression) were involved in resuscitation injury, we tested a series of novel impermeant based LVR solutions in our rodent model. We used saline as a control and all low volume resuscitation solutions were delivered at a volume equal to 10% of the calculated blood volume. We hypothesized that simple cell impermeants delivered into LVR solutions would prevent cell and tissue swelling and improve local capillary perfusion in the low volume state, which ultimately would increase the tolerance to the low volume state. This would be registered as an increase in the measured LVR times in our oxygen debt (lactate reported) models. The results of the first such series of studies is shown in Figure 5. Simple cell impermeants like raffinose,



trehalose, and gluconate, given alone or in combinations, typically can produce about a 3-4 fold increase in the LVR time in these studies. This suggests that indeed cell swelling is likely involved in the accumulation of tolerance to the low volume state because the molecules used are chemically inert and only possess known biophysical effects on compartment water and fluid movements, such as the cell and tissue swelling characteristic in ischemia. Reversal of tissue swelling with impermeant loading may increase the efficiency of capillary exchange at low volumes and explain our results. The poor results shown by sorbitol are artefactual because sorbitol can enter cells slowly but then undergo rapid conversion to fructose and then lactate by sorbitol dehydrogenase. This artificially increases lactate production in these animals by anaerobic glucose fermentation and triggers and early LVR time because the LVR time is lactate dependent. In fact, the lactates skyrockets in shocked rats receiving intravenous solutions with sorbitol. So we are unable to assess the effectiveness of sorbitol in this model because lactate synthesis is driven by substrate availability (sorbitol) and not just oxygen availability (ischemia).

In an attempt to leverage osmotic effects of impermeants in LVR solutions during shock, we tested whether addition of a second osmotic gradients imposed between the vascular and interstitial space would produce an additive or potentiative effect on the simple impermeant effect. Therefore, we added a colloidal impermeant polyethylene glycol (20,000 kDa MW, PEG-20k) to solutions with gluconate. The effects are summarized below in Figure 6. Clearly, PEG-20k added to the gluconate solution produced a marked potentiation of the gluconate effect. In fact, the magnitude of the LVR increase cannot be determined because the end lactate in the gluconate + PEG group was only 2.6 at the end of 240 minutes of low volume resuscitation, which is far lower than the 10 mM needed to trigger the official end of the LVR period. Had we waited until the plasma lactate rose to that level, the LVR time would have been much higher than 240 min. But when PEG-20k was used alone, we discovered that the effect was the same. A more comprehensive dose-response evaluation of PEG-20k as an active osmotic agent for LVR was conducted and compared to other control LVR solutions like Hespan and Albumin. The results (Figure 7) identify a minimum dose of PEG-20k of 10% blood volume infusion of a 10% weight to volume solution. Keeping the mass of PEG-20k the same by giving 5% blood volume infusion

*P<0.05, relative to all other values,
# P<0.05, relative to all other values

Figure 6

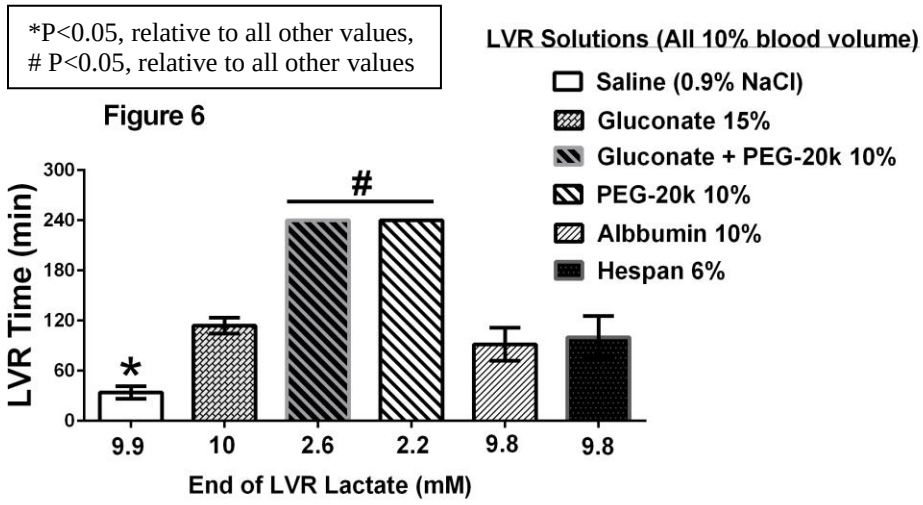
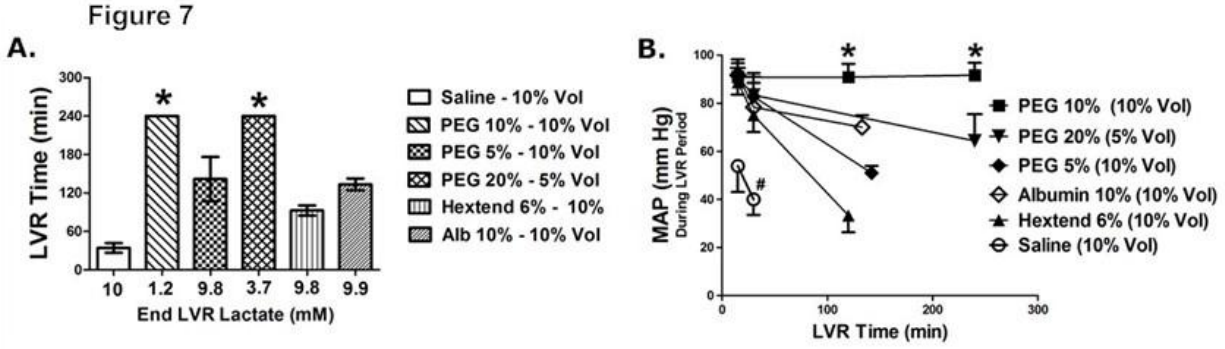


Figure 7



of a 20% weight to volume PEG-20k solution, also produces similar results and results in a very low volume solution. Furthermore, Hespan works the same as albumin and far inferior to 10% PEG-20k. In addition to impermeants expanding the LVR time (shock tolerance), they also dramatically increase

mean arterial blood pressure after low volume resuscitation (Figure 7B). The solutions with the poorest LVR times (saline and Hextend), also had the lowest MAPs during LVR. Conversely, LVR solutions containing PEG-20k had the highest LVR times and the highest MAP after LVR. The exclusive osmotic nature of this class of compounds strongly suggests that the increase in pressure is secondary to an osmotically-induced volume auto-transfusion of isotonic fluid from the cell and interstitial spaces into the capillaries. This was directly tested and data will be presented in following sections.

Twenty-four hour survival in rats after severe hemorrhagic shock, low volume resuscitation, and full resuscitation with plasma, platelets, and packed cells (1:1:1) is shown in the table. PEG-20k LVR solution was compared to an equal volume of saline (the vehicle for PEG-20k solutions).

Table. The effects of PEG-20k LVR solution on hemorrhagic shock values during the LVR period and after full resuscitation (survival)

	During LVR				
	LVR Time (min)	***MAP (mm Hg)***	***Lactate (mM)***	***HCO_3^- (mM)***	***PaO2 (mm Hg)***
Saline (10%)	34 (18)	49.3 (11)	9.53 (2.1)	11.9 (2.1)	389 (72)
PEG-20k (10%)	180 (0)*	95.0 (3.5)*	1.42 (0.6)*	25.3 (3.4)*	465 (31)
	Next Day Survival				
	Survival (%)	***MAP (mm Hg)***	***Lactate (mM)***	***HCO_3^- (mM)***	***PaO_2 (mm Hg)***
Saline (10%)	**0 (0)**	**NA**	**NA**	**NA**	**NA**
PEG-20k (10%)	100 (0)*	85.6 (6.2)*	1.2 (0.1)*	25.6 (2.6)*	475 (80)*

Values are Mean (SD), *P<0.05, Relative to corresponding saline values, n= 5, PaO2 measured with an FiO2 of 0.9.

The rats given an LVR with PEG-20k clearly had better functional and metabolic numbers during the LVR period and every one survived 24 hours later after anesthesia. By contrast, all of the saline LVR rats died during the night after anesthesia. The ones that received PEG-20k and survived, had normal arterial pressures, lactates, bicarbonate levels, and pulmonary exchange measured on the next day. These rats all acted normally and were displaying active behavior. The only values that were abnormal were related to hemoglobin, packed cell mass, and Hct because they lost 55% of their blood volume during the hemorrhage but received only a partial packed cell replacement transfusion during the full resuscitation period.

Comparisons with "Other" Solutions: The optimized solution developed for this project, which is based on an impermeant platform, has demonstrated that cell swelling is an important causal mechanism because using these agents that are known to reduce or prevent cell swelling greatly improves LVR times and outcomes in our shock models, compared to saline controls. However, since saline is not the treatment of choice anymore for shocked patients in the pre-hospital setting (11-13), we tested the impermeant based LVR solution against Hespan (14). In a further attempt to dissect out the pure colloid effects of PEG-20k in our studies from other effects, we also compared the PEG-20k effects with albumin, which is a pure oncotic agent. The responses of these solutions in our rodent LVR model are shown in **Figure 6**. Six groups of rats were shocked and the LVR times were assessed as the primary end point, which is an estimation of low volume tolerance or the "Golden Hour". In this particular trial, Polyethylene Glycol-20,000 (PEG-20k) used alone increased LVR time 8 fold more than the saline control and was at least twice as effective as Hespan or albumin, suggesting that PEG-20k probably is acting by mechanisms other than as an oncotic agent. The PEG 10% group LVR time of 240 minutes was arbitrarily determined and cut off before the lactate trigger reached 10 mM. The plasma lactate in those animals that remained in the low volume state was only 2.2 mM after 240 min. In other words, had

we waited until the lactate climbed to 10 mM, like in the other groups, the LVR time would have been significantly longer than 240 minutes. So it is clear that PEG-20k alone is much more effective than any other solution available for pre-hospital low volume resuscitation in hemorrhagic shock. It is also clear that PEG-20k, a putative oncotic agent (15, 16), is as effective by itself as it is when combined with cell impermeants such as gluconate (10). The mechanisms of how PEG-20k works in LVR is explored and reported in section IV.

III. To translate the LVR effects to a pre-clinical porcine model of hemorrhagic shock.

Until now, the effects of impermeant based solutions in low volume resuscitation injury after severe hypovolemic shock have been studied in a rodent model. Because the results are so impressive, we now attempted to translate these results into a pre-clinical large animal model that more closely represents human conditions and responses. Therefore, a similar pig model was developed to test these new osmotically based LVR solutions. The pig model is the same as the rodent model except some of the model limits were readjusted to accommodate the nature and sensitivities of the pig relative to the rat. Specifically, we dropped the lactate control points to 7-8 mM from 9-10 for the rat because pigs die soon after accumulating a plasma lactate in excess of 8 mM for short durations. When pigs reached the oxygen debt signaled by a lactate of 7-8 mM, they were given an LVR solution of either saline or PEG-20k (10% by weight) at a volume of 10% of the calculated baseline blood volume over 10 minutes. The LVR time was estimated as described before, based on changes in plasma lactate levels during and after the LVR period. Hemodynamic data were also measured in the studies. To first demonstrate that the pigs assigned to both saline and PEG groups started from the same population, we compared various experimental times during the protocol before the LVR solutions were given (Figure 8). Both groups of pigs were treated equally because attributes like surgery time, fluid loading time, time to achieve baseline, total prep time, and hemorrhagic shock time were identical in both groups. This suggests that the two populations were homogenized and only differ with regard to the treatment during the LVR period where one group received saline (civilian control solution) and the other a 10% solution of PEG-20k in saline. LVR time was the main outcome variable and is shown in Figure 8 and Figure 9B, along with plasma lactate values during the experiment (Figure 9A). In the pigs receiving the saline LVR, plasma lactate continued to rise above the 7-8 mM target even after administration of the saline bolus. We arbitrarily assigned the LVR time of 15 minutes to this group because this is the minimum time period of data collection in the study. Actually, the LVR time should be considered as zero, based on the

Figure 8

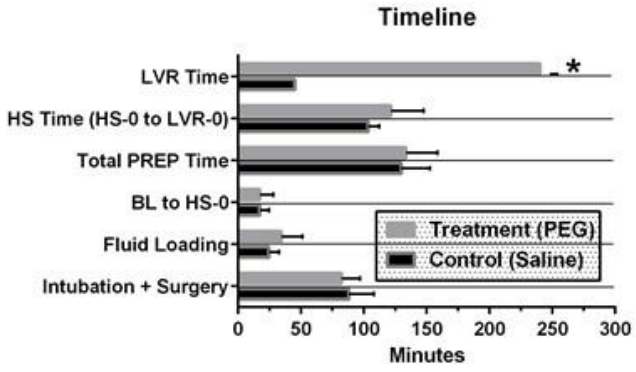
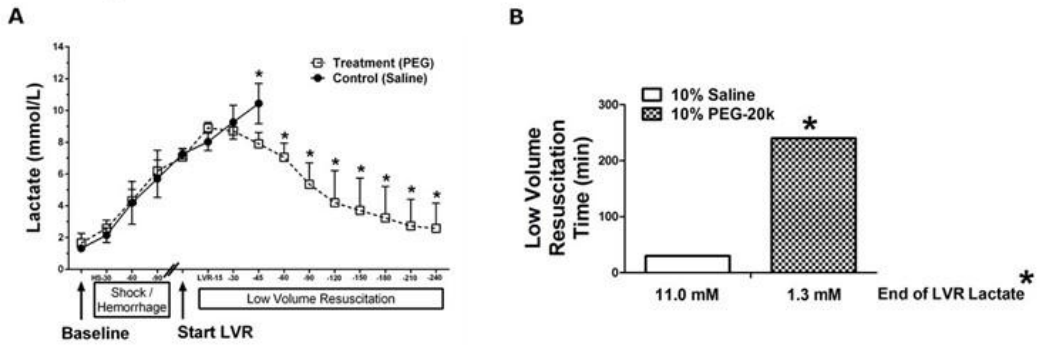


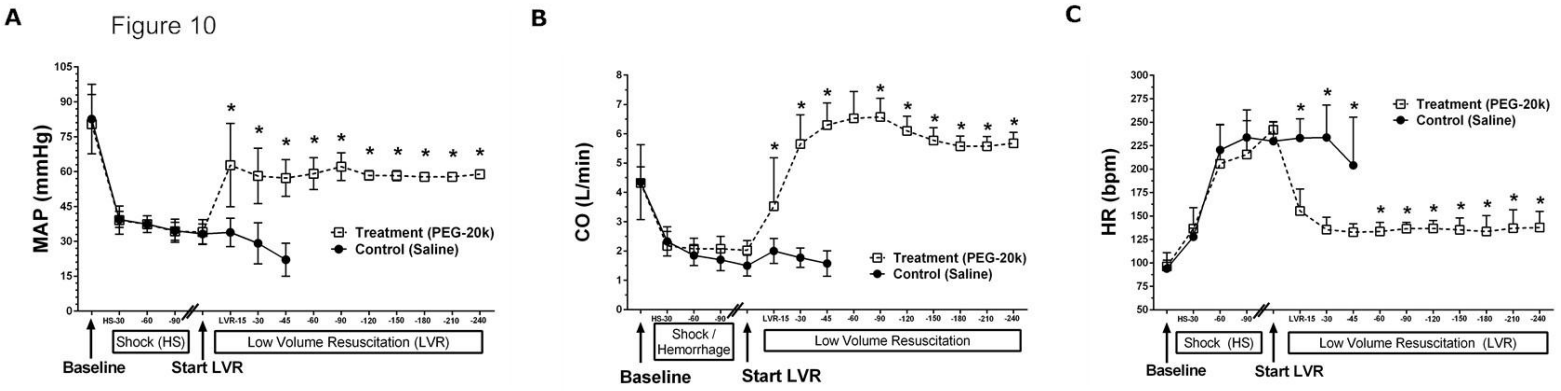
Figure 9



definition of LVR time. By contrast, plasma lactate in the PEG-20k group fell precipitously following LVR and approached baseline levels. This resulted in a calculated LVR time in the PEG-20k group of 240 minutes, which is 16 fold higher than the control group. Like the rat studies, this LVR time was arbitrary and had more to do with stopping the experiment because of anesthesia concerns for the animals because the lactate in the PEG-20k resuscitated pigs was almost normal and far away from the 7-8 mM lactate level that would trigger the end of the LVR time period. It is doubtful if the lactate would ever rise again in these animals. This will be determined when we do chronic survival studies. Therefore, pigs behave similar to rats regarding LVR time and tolerance to the low volume state when PEG-20k based LVR solutions are used to resuscitate severely shocked animals. This gives us more confidence that similar effects will be seen in human trials.

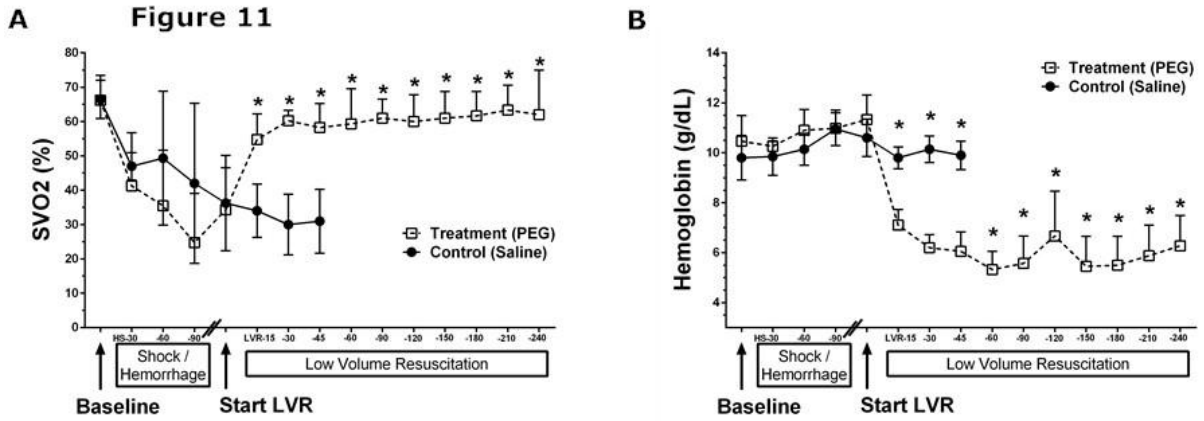
Cardiovascular outcomes were also assessed during the study in pigs. Figure 10 clearly demonstrates the differences in cardiovascular performance of the pigs after low volume resuscitation with saline compared to PEG-20k solution.

Figure 10



In the saline treated pigs, the arterial blood pressure and cardiac output fell during hemorrhage and continued to fall after LVR administration. Heart rate remained high after LVR because of the baroreceptor response to persistent hypotension. However, the same volume of PEG-20k raised MAP to a stable 60 mmHg, significantly increased cardiac output even above baseline values, and dropped the heart rate to normal as the baroreceptors reloaded in response to the increase in central blood pressure. The increase in cardiac output with PEG-20k was likely the result of increase in stroke volume due to an increase in venous return because of the volume expansion. This allowed for a hyper-dynamic cardiac output during LVR that quickly started repayment of the oxygen debt incurred during the hemorrhagic shock period. This debt repayment is seen with falling plasma lactate concentrations (Figure 9A).

The return of SVO2 and the fall in hemoglobin values seen in the PEG-20k group (Figure 11) indicate an increase in peripheral flow and blood volume, respectively, compared to saline volume controls.

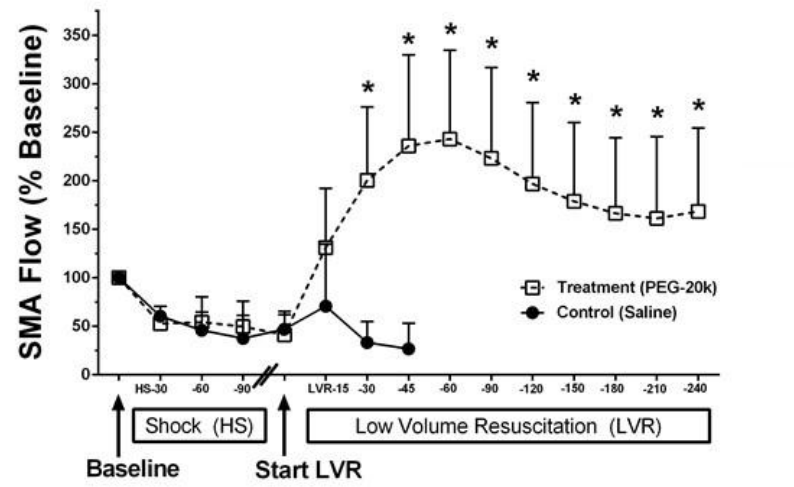


The peripheral flow in saline treated pigs continue to fall and the oxygen extraction continued to rise in an attempt to keep oxygen delivery constant. LVR using PEG-20k however, increased blood flow

through peripheral vascular beds and lowered SVO2 (Figure 11A) as the oxygen extraction dropped in the face of increased flow to maintain VO2 constant. The enhanced cardiovascular performance seems to be both by an increase in intravascular volume (which causes flow and cardiac output to rise) and a fall in peripheral vascular resistance. In this model, hemorrhage and blood loss was controlled so any changes in hemoglobin concentrations represents proportional dilution because of an inverse rise in plasma volume. This hemoglobin dilution effect is seen in Figure 11B and persists long after LVR ended. The PEG-20k is increasing isotonic water flow into the capillaries, which is the major mechanism of its action. In the saline volume controls (Figure 11B), the same IV volume had no effect on hemoglobin dilution, and therefore, no effect on volume expansion, likely because the saline was removed from the vascular space as quickly as it went in (by third spacing and capillary leak).

Maintenance of vascular volume and capillary perfusion with PEG-20k LVR solutions also seems to be associated with the maintenance of oxygen delivery to sensitive tissues including the splanchnic beds. As seen in Figure 12, low volume resuscitation with PEG-20k, but not an equal volume of saline, increased blood flow to the intestine (SMA flow) by several fold. In fact, mesenteric blood flow and oxygen delivery rose two times higher than baseline values after LVR, probably because a rapid repayment of oxygen debt was occurring. Since many have suggested that splanchnic ischemia and reperfusion may drive irreversible hemorrhagic shock, critical illness, and multipole organ failure after resuscitation, this attribute of PEG-20k based LVR solutions is appealing. The increase in capillary filling together with reduced resistance to flow in these peripheral beds leads to increased blood flow and oxygen delivery. The low resistance, compared to saline controls, likely represents a physical decompression of the capillary beds by controlling cell and tissue volumes through passive movements of water out of the tissue interstitial and intracellular spaces and into the capillary space.

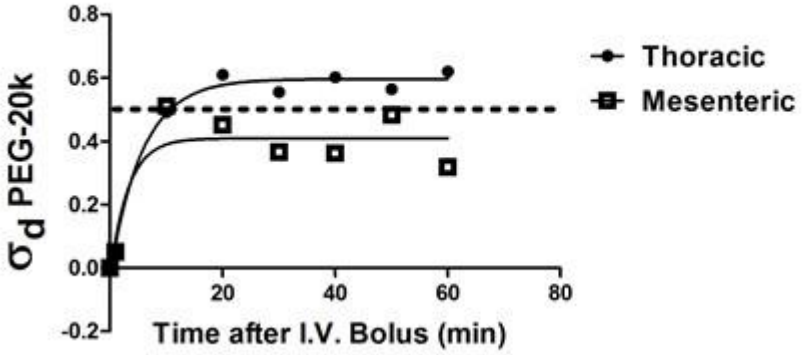
Figure 12



IV. Mechanisms of PEG-20k: The Molecular Hybrid Model: The operational hypothesis so far is that the addition of an oncotic agent to LVR solutions already containing cell impermeants would produce additive effects on the movement of water. We discovered that the LVR times doubled with cell impermeants (like gluconate) but increased 6-8 fold when the oncotic agent PEG-20k was added. In testing just the PEG-20k component alone, we discovered that it had the same effect as both combined. Further experiments revealed that pure oncotic agents like albumin were not as protective as PEG-20k. So we concluded that the striking effects of PEG-20k are not attributable solely to an oncotic action alone and that there must be something else going on with this molecule. Another observation using PEG-20k was that it caused a diuresis after administration, even in shock!. This indicated that it was permeable to capillaries in Bowman's space. These combined observations lead us to hypothesize that PEG-20-k, by nature of its unique molecular weight, may be acting as a hybrid molecule and assuming both oncotic and impermeant roles. Our data suggests that some of the PEG-20k may escape the capillary where it loads into the interstitial space (because it is impermeant to cells) and prevents cell swelling as an impermeant but some of the material stays in the capillary where it acts as an oncotic agent. This would explain our observations and suggests that impermeant solutions need only contain PEG-20k alone without specific cell impermeants like

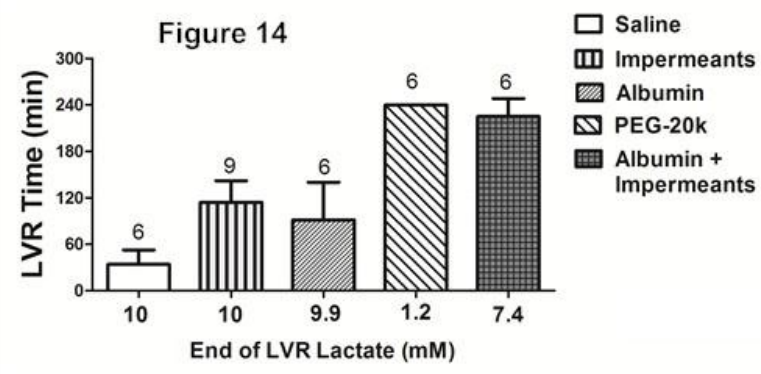
gluconate. To test this hypothesis, we measured the osmotic reflection coefficient (σ_d) for PEG-20k in normal rats. If a molecule has a reflection coefficient of 1.0, it is all reflected by the capillary wall and none escapes into the interstitial space or gets into the lymphatic system. If it has a reflection coefficient of 0, it is freely permeable to the capillary and will achieve an equilibrium between the capillary and interstitial spaces and lymphatics. To measure σ_d for PEG-20k, we cannulated the thoracic duct to collect lymph flow and then volume loaded the rat with continuous infusions of saline to increase lymph flow rate. We then administered a bolus of FITC-labelled PEG-20k and tracked the accumulation of the fluorescent label in both the plasma and lymphatic compartments. The relationship of 1-[L]/[P] of the tracer at high lymphatic flow rates is assumed to estimate the oncotic reflection coefficient (17). A representative data set plotted is shown in **Figure 13** below. Not only does the PEG-20k appear quickly in the lymph, but it has a reflection coefficient of about 0.50, which means about 1/3 of the material passes into the interstitium and about 2/3 remains in the capillary space where it acts oncotically. Therefore, these data support a compelling argument that PEG-20k is in fact a unique hybrid molecule, which explains its biological effects in shock.

Figure 13



To further support the double osmotic gradient hypothesis and the molecular hybrid behavior of PEG-20k in this setting, we designed experiments to recapitulate the PEG-20k effect in shock by using an LVR solution composed of a pure oncotic agent like albumin and a pure impermeant agent like gluconate. If PEG-20k extends the tolerance to the shock state so well because it establishes two osmotic gradients in the microcirculation (by virtue of its unique osmotic reflection coefficient), then we would predict that we should be able to mimic these effects by combining an ideal colloid with an ideal impermeant. Figure 14 shows the results of such an experiment. The impermeant effect is reproduced and the effect of gluconate alone (an impermeant) and albumin alone (an oncotic) are shown to be about equal in effect because they both produce an LVR time of about 100-120 minutes. But, when the two are added together we get an effect that is almost equal to the effect that was observed with PEG-20k alone. This supports the hypothesis that PEG-20k may be working by its hybrid nature where an impermeant and a colloidal effect are established with one molecule because we can recapitulate the biological effect by using both types of impermeant molecules together. Of course, this experiment does not rule our other completely different mechanisms of action of PEG-20k in LVR after shock but the results are consistent with the hypothesis.

Figure 14



Another prediction that may support the mechanisms of action of PEG-20k in low volume resuscitation is the reloading of the local capillaries after PEG LVR. Specifically, if PEG-20k, through its osmotic gradients created in the microcirculation, is pulling isotonic fluid from the interstitial space into the capillary, then regional capillary blood flow during LVR with PEG-20k should be much higher compared to capillary flow after a saline LVR. So we measured local capillary blood flow in rats receiving a saline LVR and a PEG-20k LVR. Using the microsphere technique, we can clearly demonstrate an increase in capillary perfusion with PEG-20k (Figure 15).

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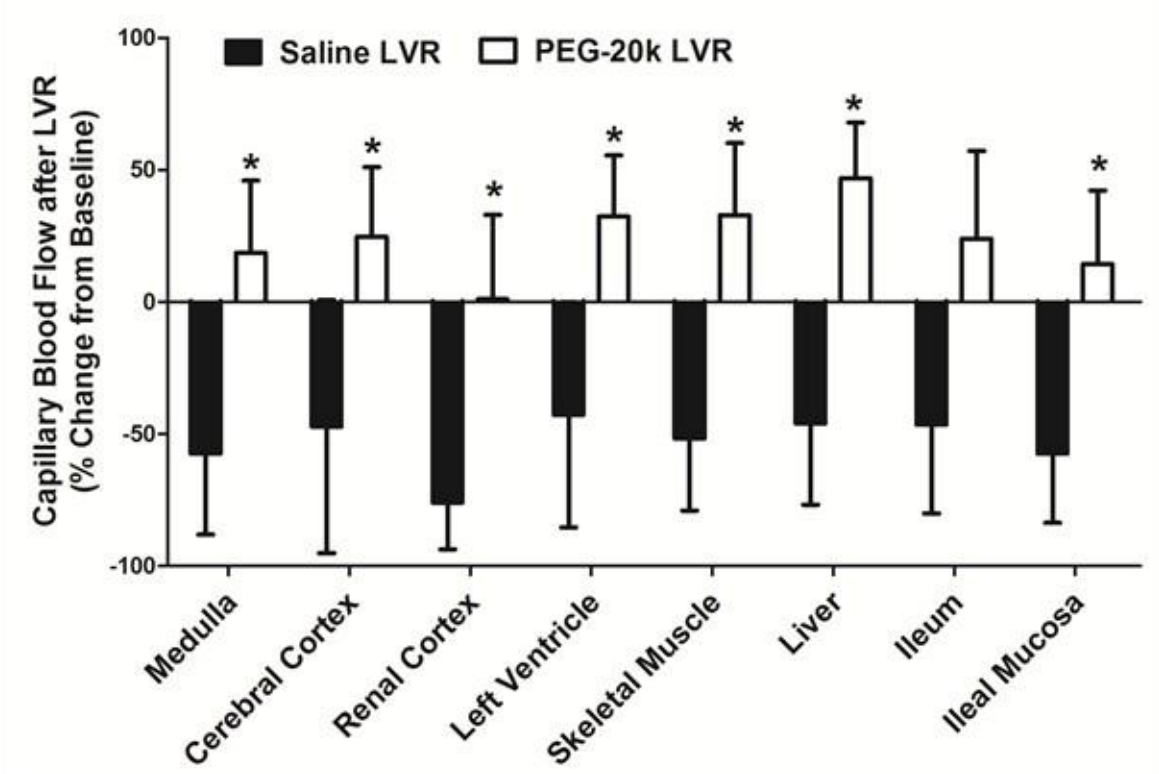
Figure 15



Capillary blood flow measured in all organs tested in the rat model of LVR shock significantly fell after shock and low volume resuscitation using a saline bolus. This occurrence was expected because the small volume of saline rapidly third spaces and prevents volume expansion in the vascular space. This also leads to a muted pressure response after saline LVR. However, PEG-20k based LVR solutions significantly increased capillary blood flow after LVR in all organs but the distal ileum. This increase in measured capillary blood flow supports the hypothesis that capillary filling is occurring after PEG-20k LVR but not after saline LVR. Again, this further supports the bigger hypothesis that PEG-20k non-energetically moves isotonic fluid from the interstitial, and intracellular spaces, where it accumulates during the low flow state, into the capillary space where it supports perfusion pressure, local capillary perfusion, and venous return.

To further test this hypothesis, we measured changes in intravascular volume in rats after severe hemorrhagic shock (55% volume bleed) and after the administration of a PEG-20k based LVR solution of a saline volume control (10% calculated blood volume for both). Figure 16 shows these changes in intra-vascular volume during the LVR period as measured using both a FITC-Albumin indicator dilution technique and by using hematocrit changes. Using RBCs as a dilution marker works because in a controlled hemorrhage model, RBCs in the vascular space stay constant during the LVR period so changes in Hct are inversely proportional to volume dilution from the direct and indirect effects of the LVR solution. Clearly, a significant dilution is seen following LVR using PEG-20k. No effect is seen with saline using the RBC technique but a small volume expansion is present when FITC-albumin is

used. The difference probably is due to albumin extravasation during shock thereby causing an apparent calculation of volume expansion when one in fact may not be occurring. This is supported by the lack of volume expansion with saline LVR seen using the RBC

Figure 16

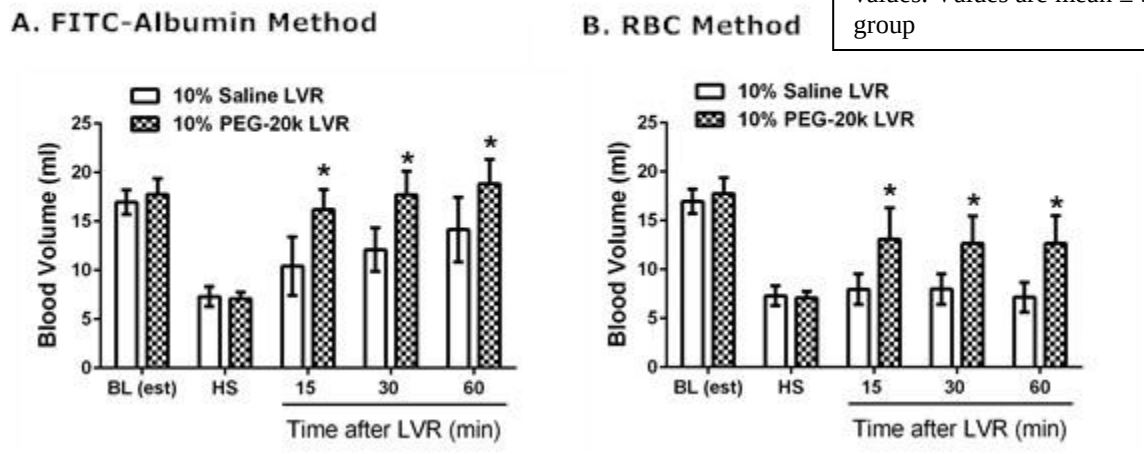
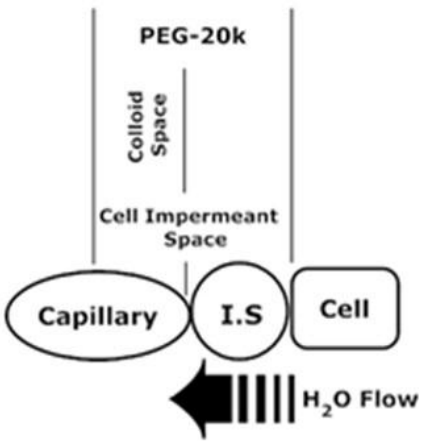


Fig 16: Baseline = BL, Hemorrhagic Shock = HS, *P<0.05 relative to corresponding saline values. Values are mean ± SD, n = 4 for each group

nique, where the RBCs are too big to extravasate to produce an apparent dilutional effect (unlike albumin). For these reasons, we believe that the RBC technique is closer to reality than the FITC-albumin technique. Bot tell us that PEG-20k LVR produces a large and significant movement of isotonic fluid into the capillary space, which again supports the osmotic movement theory and accounts for the increase in capillary blood flow, MAP, perfusion, lactate clearance, LVR time, and survival.

Based on all of these data, a simple mechanistic model illustrated in **Figure 17** is proposed to explain the strong PEG-20k LVR effect. Other possible mechanisms of PEG-20k in low volume resuscitation may include rebuilding of the endothelial and epithelial cell glycocalyx by PEG polymers. These molecules bind to cell surfaces and carry with them large amounts of organized water layers that can replace or repair the glycocalyx that erodes during the shock state. This would prevent cellular inflammatory reactions from occurring after resuscitation by camouflaging cell recognition and binding molecules on distressed cells and inducing cell passivation. However, this mechanism, even though it likely occurs, may not explain the extremely rapid effects after LVR seen in this study. The kinetics are too fast to invoke a significant cellular inflammation mechanism, but such a mechanism may contribute to later benefits in later reperfusion in chronic survival studies. We simply haven't gotten that far yet.

Figure 17



KEY RESEARCH ACCOMPLISHEMENTS:

The following significant research accomplishments include:

1. Determined the effects of oncotic and impermeant agents alone and together to increase the tolerance to the low volume state.
2. Defined a novel property of PEG-20k as a hybrid impermeant and oncotic agent with the ability to expand the golden hour up to 16 times the value observed with conventional low volume resuscitation crystalloids (saline).
3. Compared PEG-20k effects with conventional state-of-art crystalloids (Hespan and albumin).
4. Provided empirical evidence of the hybrid nature of PEG-20k in the microcirculation as indexed by the capillary oncotic reflection coefficient.
5. Demonstrated that the acute salutary effects of impermeant LVR solutions containing PEG-20k are also translatable to survival 24 hours after severe hemorrhagic shock and resuscitation.
6. Determined the preliminary safety profile of LVR solutions containing 10% PEG-20k in survival settings..
7. Translated the powerful effects observed in the rodent model to a pre-clinical porcine model.
8. Described the detailed cardiovascular effects of PEG-20k LVR in the porcine model
9. Defined the mechanism of action of impermeants and PEG-20k based low volume resuscitation solutions in providing striking tolerance to the low volume state.

IMPACT:

This project has had two major impacts in the area of resuscitation, which include; **1.)** A realignment of our thinking about the proposed mechanisms of hemorrhagic shock and resuscitation injury in tissues, organs, and organ systems and **2.)** The discovery of a highly effective pre-hospital crystalloid low volume resuscitation solution and its plethora of potential other uses in treating ischemic conditions characterized by loss of cell volume control, volume maldistribution, and microcirculatory impairment.

A large constellation of factors are believed to be involved in the current paradigm of reperfusion or resuscitation injury after prolonged ischemia, These include the destructive events secondary to the post-reperfusion synthesis of inflammatory mediator including oxygen and nitrogen free radicals and pro-inflammatory cytokines. These post-reperfusion inflammatory events involve activation and extravasation of neutrophils, monocytes, and platelets. Mediator induced vasoconstriction, cell based capillary plugging, and runaway cell death pathways are all believed to cause tissue and organ dysfunction, and a secondary cascade of terminal events resulting in massive systemic inflammation, multiple organ failure, sepsis, and death in severe shock. The treatment goal has been to find key upstream regulatory signaling circuits involved in the initial cascade where one could intervene early and stop the avalanche. Free radical scavengers, anti-inflammatory small molecules, and biologicals have all been targeted in shock and failed. The other traditional targeted approach is to prevent these events by resuscitation with agents that can increase the oxygen delivery in shock to prevent the underlying causative ischemia. While preventing ischemia or restoring oxygen delivery in shock as quickly as possible is a good idea, it often is impractical in pre-hospital settings where blood replacement is not realistic. Artificial or substitute hemoglobin carriers, perfuorocarbon laden liposomes, and a host of synthetic blood products that can be given early in the field to re-establish oxygen delivery and prevent the biochemical and cellular consequences of reperfusion and ischemia have all been tried with little success so far. Even less thought-out approaches have been tried like the infusion of hypertonic sodium chloride, which

exacerbates shock by driving sodium into ischemic cells resulting in accelerated stimulation of the sodium pump, loss of precious ATP, and amplified cell and tissue swelling. All of these approaches have failed in clinical trials so far. There are no drugs or treatments for traumatic shock and resuscitation injury after all of this effort because we have been targeting incorrect or largely non-significant mechanisms. This proposal and its body of work over the last 3 years (and previous years before), should realign how we need to think about causal factors of resuscitation injury in severely shocked and metabolically stressed patients. The rather impressive success of this largely osmotic approach not only opens up doors for developing truly effective low volume resuscitation solutions for pre-hospital use, but it draws a big circle around truly important causal or contributory mechanisms of resuscitation injury that have been either overlooked all together or dismissed as minor events. We now should accept that ischemia-induced loss of cell and tissue volume control pathways plays a crucial role. This is true because specific agents that effectively reverse isotonic water flow in shock dramatically mitigate the cardiovascular and metabolic injury by increasing the microcirculatory exchange of oxygen in the tissues during severe low volume conditions. This is tolerance induction to the low volume state. These data tell us that capillary no-reflow is much more important in causing resuscitation injury than all of these other factors from the last 50 years. While this will be dismissed by many, especially those invested in other mechanisms, the data are so strong and clear that to ignore its significance is to ignore the obvious. Our mechanistic studies clearly support this hypothesis, which has significant impact on how we should mechanistically see shock and resuscitation injury in tissues and organs, not exclusively but in large part. Finally, the obvious second impact is the clinical utility of these new solutions in providing life-saving resuscitation with extremely low volume. Low volume resuscitation solutions made around these impermeants are stable and effective. One 500-ml bag of solution can stabilize a severely shocked patient's cardiovascular and bioenergetic status that is on the edge of collapse and decompensation. These solutions are over 18 fold more effective than the same volume of saline or Hextend at maintaining the subject in the low volume state until definitive resuscitation can occur. This will obviously have a huge impact on pre-hospital resuscitation in military and civilian settings. Countless other applications exist wherever cell and tissue ischemia cause volume control defects, cell and tissue swelling, and no-reflow. These conditions include: Emergency Department (ED) volume replacement, CPR resuscitation in the ED, intra-operative volume replacement and readjustments, priming of cardiopulmonary bypass circuits, volume replacement and volume readjustments in the surgical ICU, severe burn shock resuscitation in the burn ICU, cardiovascular support and volume readjustments in critical illness and high output septic shock, hemodynamic stabilization of organ donors in the ICU, volume readjustments and brain swelling in traumatic brain injury, and in the neurosurgical ICU.

CHANGES / PROBLEMS: There were no significant changes or problems over the project period.

PRODUCTS:

1. Four full length publications in peer reviewed journals in 2 years.

    - Cell impermeant based low volume resuscitation in hemorrhagic shock: A biological basis for injury involving cell swelling.
      Parrish D, Lindell S, Reichstetter H, Aboutanos M, Mangino MJ. AnnSurg. Mar;263 (3):565-72, 2016
    - New low-volume resuscitation solutions containing PEG-20k.
      Parrish D, Plant V, Lindell SL, Limkemann A, Reichstetter H, Aboutanos M, Mangino MJ. J Trauma Acute Care Surg. Jul;79 (1):22-9, 2015
    - Low volume resuscitation for hemorrhagic shock: Understanding the mechanisms of PEG-20k
      Plant V, Parrish D, Limkemann A, Ferrada P, Aboutanos M, Mangino MJ
      Shock (in press)
    - Low volume resuscitation using polyethylene glycol-20k in a pre-clinical porcine model of hemorrhagic shock

Plant V, Limkemann A, Liebrecht L, Blocher C, Ferrada P, Aboutanos M, Mangino MJ J. Trauma Acute Care Surg. (in press)

2. Consecutive presentations of results at **EAST** (Eastern Association of the Surgery for Trauma) in 2015, **AAST** (American Association for the Surgery of Trauma) in 2015, **WTS** (Western Trauma Society) in 2016. This is extremely difficult to accomplish.
3. Numerous presentations of these data at local, state, and regional competitions of the Committee on Trauma (COT).
4. Three presentations at the Humera Society, a local Virginia surgical society.
5. One US Patent with PCT and CIP filings
6. One product development analysis by Global Pharmaceuticals, Inc. for the development and approval strategies for FDA 510k and NDA approval.
7. Training of (3) surgical residents, (2) post-doctoral fellow, and (2) graduate students in state-of-the-art shock and trauma research.

PARTICIPANTS:

- Martin J. Mangino, PhD. (PI)
- Susanne Lindell, BSN (Technician)
- Tao Tian, PhD (Staff Scientist)
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- Dan Parrish, MD (General Surgery resident)
- Ashley Limkemann, MD (General Surgery resident)
- Chuck Blocher, BS (Animal Technician)
- Valerie Plant, MD (General Surgery resident)
- Loren Liebrecht, MD (Post-doctoral fellow)
- Clementina Ramos, MD (Post-doctoral Fellow)
- Rahul Anand, MD (Trauma Surgeon)
- Ajai Malhotra, MD (Trauma Surgeon)

SPECIAL REPORTING REQUIREMENTS: None

CONCLUSIONS:

1. Cell and tissue swelling is a significant causal mediator of resuscitation injury after severe hemorrhagic shock.
2. Cell impermeants based LVR solutions containing PEG-20k increase tolerance to the low flow state (golden hour) 8-20 fold more than saline or Hespan volume controls.
3. Cell impermeants based LVR solutions containing PEG-20k increase survival 100%, relative to saline in rodents.
4. PEG-20k based LVR solutions significantly increase mean arterial blood pressure in the low volume state.
5. PEG-20k based LVR solutions significantly increase capillary blood flow to all organ beds in the low volume state.

6. PEG-20k can be effective alone in LVR solutions because it behaves as a hybrid molecule expressing both cell impermeant and colloid attributes together.
7. PEG-20k has an osmotic reflection coefficient in mesenteric and thoracic beds of about 0.5
8. Simultaneous use of a pure colloid (albumin) with a pure extracellular impermeant (gluconate) emulates PEG-20k alone.
9. PEG-20k based cell impermeants appear safe as used in LVR solutions in rodents.
10. PEG-20k LVR solutions are highly effective in severe hemorrhagic shock in pre-clinical porcine models because they;
    - Increase the LVR time indefinitely
    - Increase mean arterial pressure to about 60 mmHg during the low volume state
    - Cause autotransfusion of isotonic fluid from the extravascular space into the capillary space
    - Rapidly clears and normalizes plasma lactate concentrations after LVR
    - Restarts aerobic metabolism after LVR
    - Increases cardiac output and oxygen delivery after LVR
    - Increases splanchnic blood flow during LVR
    - Starts repayment of the oxygen debt during the low volume state.

BIBLIOGRAPHY (produced from the project):

Full Length Papers:

1. **Cell impermeant based low volume resuscitation in hemorrhagic shock: A biological basis for injury involving cell swelling.**
   Parrish D, Lindell S, Reichstetter H, Aboutanos M, Mangino MJ. Ann Surg. Mar;263 (3):565-72, 2016
2. **New low-volume resuscitation solutions containing PEG-20k.**
   Parrish D, Plant V, Lindell SL, Limkemann A, Reichstetter H, Aboutanos M, Mangino MJ.
   J Trauma Acute Care Surg. Jul;79 (1):22-9, 2015
3. **Low volume resuscitation for hemorrhagic shock: Understanding the mechanisms of PEG-20k**
   Plant V, Parrish D, Limkemann A, Ferrada P, Aboutanos M, Mangino MJ
   Shock (in press)
4. **Low volume resuscitation using polyethylene glycol-20k in a pre-clinical porcine model of hemorrhagic shock**
   Plant V, Limkemann A, Liebrecht L, Blocher C, Ferrada P, Aboutanos M, Mangino MJ
   J. Trauma Acute Care Surg. (in press)

Abstracts:

1. **Salutary Effects of Cell Impermeants in Hemorrhagic Shock**
   Mangino MJ, Lindell SL, Muir H, Malhotra A, Ivatury R.
   Advanced Technology Applications to Combat Casualty Care (ATACCC) 2011
2. **Low Volume Resuscitation for Hemorrhagic Shock: Understanding the Mechanism of PEG-20k**
   Plant P, Lindell SL Reichstetter H, Aboutanos M, Parrish D, Limkemann A, Ferrada P, Mangino MJ.
   American Association for the Surgery of Trauma (AAST) 2015
3. **Cell impermeants improve outcomes in low volume resuscitation for hemorrhagic shock**.
   Parrish D, Plant V, Limkemann A, Reichstetter H, Aboutanos M, Mangino MJ.

Eastern Association for the Surgery of Trauma (EAST) 2015

4. **Low Volume Resuscitation Using Polyethylene Glycol-20k in a Pre-clinical Porcine Model of Hemorrhagic Shock.**
   Plant V, Liebrecht L, Limkemann A, Blocher C, Ferrada P, Aboutanos M, Mangino MJ.
   Western Trauma Association (WEST) 2016

Presentations:

1. **Salutary Effects of Cell Impermeants in Hemorrhagic Shock**
   Mangino MJ, Lindell SL, Muir H, Malhotra A, Ivatury R.
   Advanced Technology Applications to Combat Casualty Care (ATACCC) 2011
   Poster Presentation

2. **Low Volume Resuscitation for Hemorrhagic Shock: Understanding the Mechanism of PEG-20k**
   Plant P, Lindell SL Reichstetter H, Aboutanos M, Parrish D, Limkemann A, Ferrada P, Mangino MJ.
   American Association for the Surgery of Trauma (AAST) 2015
   Oral Talk

3. **Cell impermeants improve outcomes in low volume resuscitation for hemorrhagic shock**.
   Parrish D, Plant V, Limkemann A, Reichstetter H, Aboutanos M, Mangino MJ.
   Eastern Association for the Surgery of Trauma (EAST) 2015
   Oral Talk

4. **Low Volume Resuscitation Using Polyethylene Glycol-20k in a Pre-clinical Porcine Model of Hemorrhagic Shock**.
   Plant V, Liebrecht L, Limkemann A, Blocher C, Ferrada P, Aboutanos M, Mangino MJ.
   Western Trauma Association (WEST) 2016
   Oral Talk

5. **Low Volume Resuscitation with Cell Impermeants**
   Mangino MJ
   US Army Medical Research and Material Command: In Progress Review
   DoD Hemorrhage and Resuscitation Research and Development Program
   Metabolic and Tissue Stabolization 2013
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APPENDIX:

Copy of (4) published papers and accepted manuscripts for publication

1. **Cell impermeant based low volume resuscitation in hemorrhagic shock: A biological basis for injury involving cell swelling.**
   Parrish D, Lindell S, Reichstetter H, Aboutanos M, Mangino MJ. AnnSurg. Mar;263 (3):565-72, 2016
2. **New low-volume resuscitation solutions containing PEG-20k.**
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   J Trauma Acute Care Surg. Jul;79 (1):22-9, 2015
3. **Low volume resuscitation for hemorrhagic shock: Understanding the mechanisms of PEG-20k**
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   Plant V, Limkemann A, Liebrecht L, Blocher C, Ferrada P, Aboutanos M, Mangino MJ
   J. Trauma Acute Care Surg. (in press)

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Appendix

Cell Impermeant-based Low-volume Resuscitation in Hemorrhagic Shock

A Biological Basis for Injury Involving Cell Swelling

Dan Parrish, MD, Susanne L. Lindell, BSN,* Heather Reichstetter, LVT,* Michel Aboutanos, MD,* and Martin J. Mangino, PhD*†‡*

Objective: To determine the role of cell swelling in severe hemorrhagic shock and resuscitation injury.

Background: Circulatory shock induces the loss of energy-dependent volume control mechanisms. As water enters ischemic cells, they swell, die, and compress nearby vascular structures, which further aggravates ischemia by reducing local microcirculatory flow and oxygenation. Loading the interstitial space with cell impermeant molecules prevents water movement into the cell by passive biophysical osmotic effects, which prevents swelling injury and no-reflow.

Methods: Adult rats were hemorrhaged to a pressure of 30 to 35 mmHg, held there until the plasma lactate reached 10 mM, and given a low-volume resuscitation (LVR) (10%–20% blood volume) with saline or various cell impermeants (sorbitol, raffinose, trehalose, gluconate, and polyethylene glycol-20k (PEG-20k). When lactate again reached 10 mM after LVR, full resuscitation was started with crystalloid and red cells. One hour after full resuscitation, the rats were euthanized. Capillary blood flow was measured by the colored microsphere technique.

Results: Impermeants prevented ischemia-induced cell swelling in liver tissue and dramatically improved LVR outcomes in shocked rats. Small cell impermeants and PEG-20k in LVR solutions increased tolerance to the low flow state by two and fivefold, respectively, normalized arterial pressure during LVR, and lowered plasma lactate after full resuscitation, relative to saline. This was accompanied by higher capillary blood flow with cell impermeants.

Conclusions: Ischemia-induced lethal cell swelling during hemorrhagic shock is a key mediator of resuscitation injury, which can be prevented by cell impermeants in low-volume resuscitation solutions.

Keywords: gluconate, Ischemia, osmotic effects, polyethylene glycol, resuscitation

(*Ann Surg* 2015;00:1–8)

Deaths due to injury in the United States reached more than 171,000 and costs more than $400 billion a year in health care costs and lost productivity in 2010.[1] Deaths from trauma are the number 1 cause of death for people younger than 44 years in the United States and the third leading cause of death overall for all age groups. Trauma accounts for about 30% of all life years lost in the United States, compared to cancer (16%), heart disease (12%), and human immunodeficiency virus (2%).[2] For all traumatic injuries, hemorrhagic shock is responsible for more than 35% of prehospital deaths and more than 40% of all deaths within the first 24 hours. This is second only to trauma deaths induced by severe central nervous system injury.[3] Finally, hemorrhagic hypotension exposes the patient to immediate complications of life-threatening infections, coagulopathies, and multiple organ failure.[4,5]

Early resuscitation strategies include the use of low volumes of intravenous blood products to increase oxygen delivery and to replace lost coagulation and clotting factors (coagulation proteins and platelets). Although this approach is fine for hospital emergency departments, it is not currently practical in prehospital settings where early intervention may be the key to preventing future complications following more definitive resuscitation. Crystalloids are available for prehospital use because they can be safely transported and stored but they are generally limited in their effectiveness. Attempts to modify basic intravenous crystalloids for prehospital resuscitation by adding hypertonic NaCl or starch (Hextend) as a volume expander have had disappointing results.[6,7] The future use of effective spray dried blood products will be a valuable tool in prehospital settings because they replace chemical coagulation precursors and factors. The use of fresh frozen plasma in the field, which is currently being tested at many centers, will also be useful but it too is limited by the need for refrigeration. There remains a need for a better crystalloid to resuscitate patients with severe hemorrhagic shock, especially in a prehospital setting. The successful design of such a solution is highly dependent on understanding the pathophysiological mechanisms that lead to injury during hemorrhagic hypotension and subsequent resuscitation. The optimal solution will likely be an effective new stable crystalloid that targets these mechanisms used together with reconstituted dried plasma products for the replacement and reconstitution of coagulation potential.

The predominant root mechanism of injury in hemorrhagic shock is energy failure. Although global ischemia and reperfusion injury are causally based at many levels, they all arise from changes that occur when the cell energetics drops because of a loss of adequate microvascular oxygen transport and subsequent loss of aerobically produced high-energy adenine nucleotides.[8–10] One mechanism of cell, tissue, and organ injury is cell swelling that occurs from the loss of adenosine triphosphate (ATP)-dependent cell volume regulatory control mechanisms. In most cells, the single highest energy-consuming process is the running of the Na/K ATPase pumps in the cell membrane. These pumps actively transport sodium ions out of the cell to maintain membrane potentials and to run numerous Na^+-dependent facilitated membrane transport processes such as calcium, glucose, amino acids, and organic cation transporters. In the absence of ATP to run those pumps, as occurs in ischemia after hemorrhagic shock, the Na/K ATPase turns off and sodium enters the cell as it runs back down its electrochemical gradient. The elevated intracellular sodium futilely stimulates the sodium pump that cannot run because of loss

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of ATP.[11] Chloride then enters the cell down an electrical gradient and water follows the sodium chloride down a developing osmotic gradient, which causes the cell to swell. Hydropic degeneration from energy failure damages membrane and mitochondrial structures,[12] which may lead to cell death.

This basic mechanism of cell ischemic injury has been well described in organ preservation associated with transplantation.[13–15] Effective modern organ preservation solutions were developed around this concept and contain high concentrations of cell impermeants.[16] These are classes of nontoxic molecules, usually saccharides and small organic cations and anions, which are small enough to freely egress the capillary space in the microcirculation but are too large or too charged to cross the cell membrane. As such, they preferentially load into the interstitial space where they create an osmotic force that prevents the movement of water into the cell as the sodium concentrations rise during ischemia. They prevent lethal cell swelling. Cell impermeant, as a class of agents, are one of the most effective components of organ preservation solutions used today.[17] The University of Wisconsin solution contains high amounts of raffinose, lactobionic acid, sulfate, and phosphate, which all act as cell impermeants to prevent water movement. The Belzer-UW MPS solution uses gluconate and histadine-tryptophan-ketogluterate solution uses both high concentrations of histidine and mannitol as impermeants. Histidine at physiological pH is charged and is an impermeant. Water movement in organ preservation is slower than ischemia at normal mammalian temperatures because hypothermia is used to preserve organs, which slows down the process. Because cell swelling during ischemia induced by hemorrhagic hypotension also occurs[18] and at a much faster rate than in organ preservation because of the warmer temperatures, it was hypothesized that loading the interstitial space with nontoxic cell impermeants during the low-volume period would prevent lethal cell swelling and increase the tolerance of the patient to the low-volume state and improve outcomes at resuscitation. Testing this was the objective of the study.

METHODS

All animal work was conducted under a protocol approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee, which is governed by the rules and regulations set forth in the National Institutes of Health guide and the United States Department of Agriculture.

In Vitro Model

Warm ischemia-induced cell swelling and the effects of cell impermeants on this response was first characterized in mouse liver slices. Liver slices have been used before to characterize cell impermeants[19] because they are easy to prepare and there is abundant mass for many groups per liver. Adult mice (C57BL/6) were anesthetized with isoflurane and the liver was isolated, quickly removed, and immersed in cold saline on ice. Liver slices (3–4 per condition) were prepared with a Staddie-Riggs microtome to give a uniform thickness of less than 0.5 mm. About 150 mg of liver slices were incubated in 25-mL Erlenmeyer flasks in 1.5 mL Krebs buffer in a Dubanoff style metabolic shaking water bath under an atmosphere of oxygen or nitrogen always containing 5% CO_2. Tissue slices underwent ischemia by incubation under an atmosphere of 95% nitrogen and 5% CO_2 for 1 hour followed by reperfusion under an atmosphere of 95% oxygen and 5% CO_2 for an additional hour. Some tissue slice conditions contained impermeants in the Krebs buffer during ischemia and some did not (controls). Impermeants were used at 0, 25, 50, 100, and 150 mM final concentration. These impermeants consisted of sorbitol, gluconate, trehalose, raffinose, and an equal molar mixture of raffinose and trehalose. Tissue slices were sampled after preparation (Fresh), after ischemia, and after reperfusion in untreated and impermeant treated groups for analysis of total tissue water (TTW) content by calculating [wet-dry]/dry weight ratios. Dry weights were determined after drying the tissue slices in a 65°C oven for 48 hours.

Rodent Shock Model

A low-volume resuscitation (LVR) model was used in adult rats to test both the cell swelling hypothesis and to develop the impermeant-based LVR solution used for prehospital resuscitation of patients with severe hemorrhagic shock. Adult Sprague Dawley rats were anesthetized with isoflurane and maintained in a light surgical plane of anesthesia during the study. Polyethylene catheters were placed in both femoral arteries for blood pressure monitoring and blood sampling, and a catheter was placed in 1 femoral vein for administration of fluids. The animals were allowed to ventilate on their own to establish normal arterial blood gas (ABG) values. A 1-cm midline incision was created to induce soft tissue injury and for the placement of a temperature probe in the abdomen. The animals were kept at 38°C using a heating pad and an incandescent light source above them. Arterial blood pressure, heart rate, and temperature were continuously recorded using a PowerLab (ADInstruments, Boston, MA). After a 30-minute stabilization period, heparin was given (500 U/kg) and arterial blood was slowly removed at 1 mL/min into a syringe to maintain blood pressure at 30 to 35 mm Hg. This hypotension was maintained until the plasma lactate reached a value between 9 to 10 mM, as measured with both a handheld lactate analyzer (Lactate Plus, Nova Biomedical, Waltham, MA) and a blood gas analyzer (Radiometer 800). Once the target lactate was reached, an LVR equal to 10% to 20% of the calculated blood volume[20] of saline was administered intravenously over a 10-minute period using a syringe infusion pump. When the blood lactate again reached 9 to 10 mM, full resuscitation was started, which consisted of a volume of saline equal to the volume of the blood loss (about 55% to 60% of total blood volume) plus 30% of the removed red blood cells (washed) infused intravenously over 10 minutes (although this full resuscitation protocol using saline is now outdated, the study was started when it was acceptable to use saline so the authors finished the project using the same protocol). After 1 hour of full resuscitation, the animals were euthanized by an anesthetic overdose and terminal blood was removed for analysis. The time from the start of the LVR period until the start of full resuscitation is called the LVR time and it represents the tolerance of the animal to the low-volume state or the maximum amount of time that a shocked subject can safely remain in the low-volume state until more definitive resuscitation is required. This was a major outcome used in the study. The protocol is illustrated in Figure 1.

Regional Blood Flow

In another series of studies ($n = 6$ per group), the effects of hemorrhagic shock, LVR, and LVR with impermeants on local capillary blood flow were studied using the colored microsphere technique.[21,22] Animals were prepared as previously described but a catheter was also placed into the left ventricle by advancing the catheter through the right carotid artery using real time pressure and pressure waveforms as indicators of the catheter location. Once all catheters were in place, 0.2 mL colored microspheres (Triton Technologies, San Diego, CA) were rapidly injected into the left ventricle. A calibrated arterial reference blood sample was simultaneously removed from the femoral artery catheter by a withdrawal pump to calibrate the microsphere measurement. Three different microsphere colors were used at baseline, during LVR (immediately before full resuscitation), and 60 minutes after full resuscitation. After the study, tissue samples were removed from major organs and the microspheres

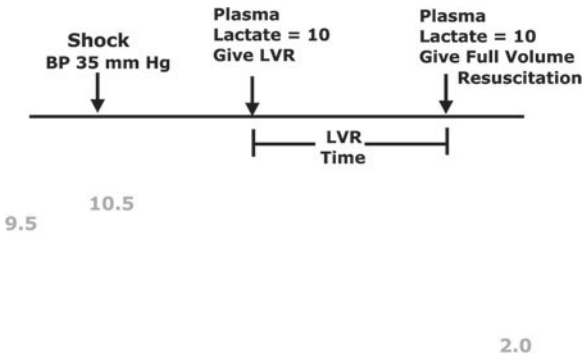

FIGURE 1. The LVR protocol that was used in these studies. Arterial hemorrhage is used to maintain a low-volume state (30–35 mm Hg) until the plasma lactate reaches 9 to 10 mM. At that time, an LVR solution is given, which temporarily reduces plasma lactate (due to dilution and increased perfusion) and increases arterial blood pressure. When lactate again reaches 10 mM, full resuscitation is started with 1 volume of saline containing 30% of the washed red cells that were hemorrhaged. The time from the start of the LVR solution until the start of the full resuscitation solution is called the LVR time and it represents the tolerance of the patient to the low-volume state or the maximum amount of time that a patient can safely remain in the low-volume state until more definitive medical care and full resuscitation can be started. The LVR time was a key outcome variable for these studies.

in the tissues and in the reference arterial blood samples were recovered by alkaline digestion and repeated centrifugations. Dye coating the purified microspheres was extracted with dimethylformamide and quantitated using a UV-VIS spectrophotometer (Shimadzu). Individual colors were resolved using a matrix inversion algorithm from the composite spectra. Blood flow was calculated by the tissue dye content using the reference blood draw as a standard.

Experimental Design

Shocked animals were treated according to the following groups:

1. Saline Controls: Received saline as the LVR solution (n = 12).
2. Gluconate: Received an LVR solution of 15% gluconate in saline, a prototypical cell impermeant (n = 11).
3. Gluconate + PEG-20k: Received an LVR solution of 15% gluconate and 10% polyethylene glycol with a molecular weight of 20 kDa (PEG-20k). PEG-20k acts as an oncotic agent (n = 8).
4. PEG-20k: Received an LVR solution of 10% PEG-20k (n = 6).
5. BSA: Received an LVR solution of 10% Bovine Serum Albumin (BSA), a prototypical oncotic agent (n = 6).

The outcome variables for the study included LVR time, plasma lactate, mean arterial blood pressure, and regional tissue blood flow rates.

Statistical Analysis

Most data are expressed as the group mean ± the standard deviation. Each group consisted of 6 to 12 subjects per group, which was derived from power analysis and the known variance of the data in the studies. Data were analyzed by analysis of variance and Bonferroni's multiple comparison test. All data were first analyzed for normality of distribution. A $P < 0.05$ was considered statistically significant.

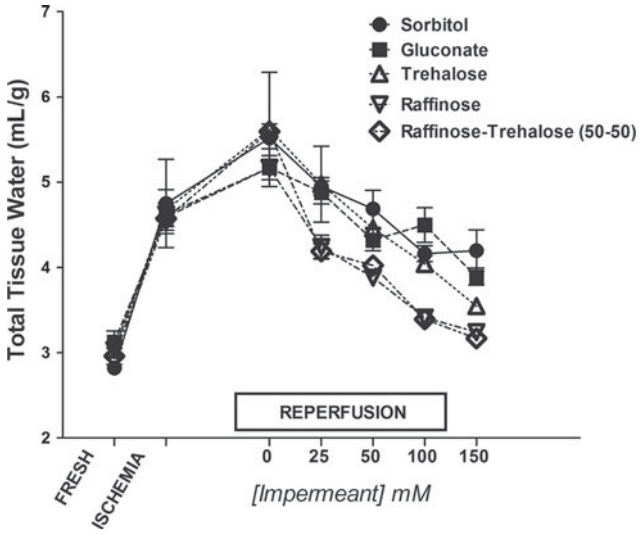

FIGURE 2. Cell swelling of liver tissue slices in vitro in response to hypoxic ischemia and the effects of various concentrations of cell impermeants on the cell swelling response. Cell swelling was indexed by measuring TTW of the liver slices an hour after ischemia and an hour after normoxic reperfusion or in fresh controls. Cell impermeants were in the Krebs buffer suffusing the slices during ischemia. In general, the impermeant effect is proportional to the molecular weight of the impermeant and its concentration in the extracellular space. n = 6 liver samples per group, values are mean ± SD. Each impermeant group also has a zero concentration control, which sees ischemia and reperfusion without any impermeants.

RESULTS

The impermeant effects of a variety of common cell impermeants in the in-vitro tissue slice model is shown in Figure 2. TTW measurements indicate that 60 minutes of hypoxic ischemia to murine liver slices caused tissue water accumulation to increase almost twofold after ischemia alone and after 1 hour of normoxic reperfusion. The addition of all of the molecular species of cell impermeants to the incubation media during ischemia prevented the ischemia-induced water accumulation after reperfusion. The magnitude of the response was generally directly proportional to both the molecular weight of the impermeant and the molar concentration in the media (25–150 mM). The optimal responses were observed with raffinose and mixtures of raffinose and trehalose used at about 60 to 100 mM.

The amount of time that a shocked subject can safely remain in the low-volume state is indexed by the LVR time in this experiment. These times are shown in Figure 3 for the various treated groups of shocked rats. The trigger to end the LVR period after the LVR solutions were given was the lactate climbing back up to 9 to 10 mM. Gluconate (15%) added to the saline-control LVR solution increased the LVR time by 100% from about 45 minutes for the saline control to more than 96 minutes for the gluconate solution. The addition of 10% PEG-20k to the gluconate LVR solution further increased the LVR time 5.3-fold over the saline control to 240 minutes. This LVR time was arbitrarily stopped because of anesthesia effects but it likely could have gone much longer because the target lactate of 10 mM was never reached even after 240 minutes after the start of the LVR solution. The lactate in the gluconate + PEG-20k group after 240 minutes was only 2.5 mM. Similarly, the LVR time in the group

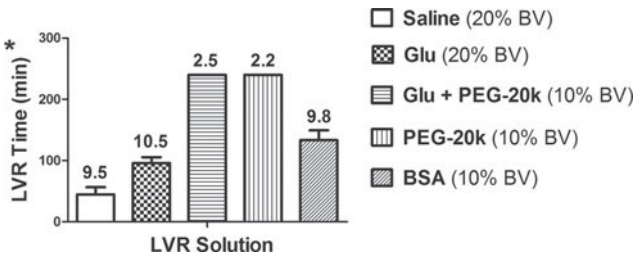

FIGURE 3. The LVR time measured in 5 groups of shocked rats. The LVR time is the time from the start of the LVR until the time of full resuscitation, based on the response of the plasma lactate levels. The values are mean ± SD with 6 to 12 animals per group. The numbers above the bars are the average plasma lactate value at the end of LVR. Because 10 was the cutoff to end LVR by definition, most of the lactate values are very close to 10. However, the 2 groups with PEG-20k in the LVR solution were arbitrarily ended at an LVR time of 240 minutes and the ending lactate values were still far below the target of 10 mM. The standard LVR volume was chosen to be 20% of the calculated blood volume but this had to be cut in half for the PEG-20k groups because the response and diuresis were too intense. $^{*}P < 0.05$ between all groups except between the 2 groups with PEG-20k, GLU = sodium gluconate.

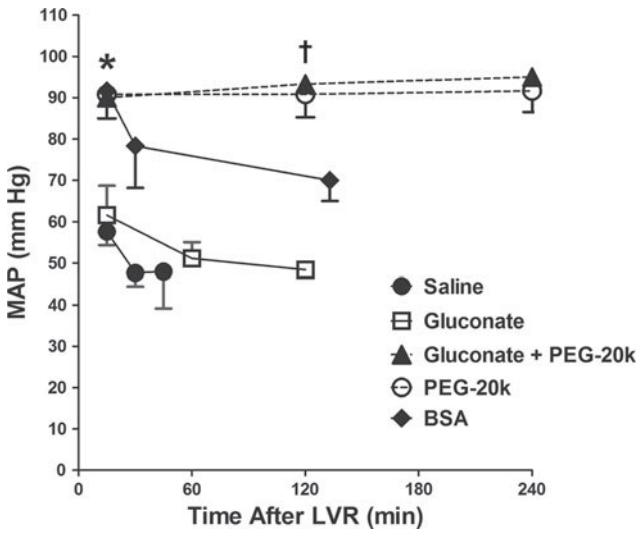

FIGURE 4. MAP in animals during the LVR time after hemorrhagic shock in the 5 groups of shocked rats. Values are mean ± SD, n = 6–12 per group, $^{*}P < 0.05$ relative to the gluconate and saline groups, $\dagger P < 0.05$, relative to the BSA and gluconate groups (at the same approximate time point).

with only PEG-20k was also ended after 240 minutes with a lactate of only 2.2 mM. Because the time limit of these 2 groups was never met because the target lactates were never met, we do not know if there is in fact a difference in the 2 groups with respect to LVR time. Finally, LVR solutions containing 10% BSA were also effective at increasing the LVR time (133 minutes) but not nearly as effective as LVR solutions containing PEG-20k. It is also important to note that the volume of LVR solution used that contained PEG-20k was half the volume (10%) used in the other groups (20%: saline control, gluconate, and BSA). Thus, PEG-20k based LVR solutions were more than 5 times more effective at expanding the LVR time compared to saline, at half the dose.

The mean arterial blood pressure in rats after shock and after administration of the LVR solution (for as long as the LVR period lasted) is shown in Figure 4. In the saline controls, the blood pressure after the shock period was 30 to 35 mm Hg, by definition of the model. After 10 minutes of saline LVR administration, the mean arterial pressure (MAP) rose initially to about 55 mm Hg but then rapidly fell back below 50 mm Hg as the LVR period ended after 45 minutes, because of the lactate reaching 10 mM. The gluconate group showed a similar pattern. Although the MAP did not get higher than the control group, it did last longer because gluconate doubled the LVR time. Groups resuscitated with PEG-20k in the LVR solution, however, had normal MAP throughout the 240 min LVR period and this was accomplished with only 50% of the LVR resuscitation volume of the controls. The BSA-treated oncotic controls started with a normal blood pressure immediately after LVR solution administration, which fell off to about 70 mm Hg at the end of the LVR period. This was significantly higher than the control MAP but significantly lower than the MAP for the groups resuscitated with LVR solutions containing PEG-20k.

Figure 5 shows the final plasma lactate levels in shocked rats after LVR and 1 hour after full resuscitation. The lactate levels were all significantly lower in animals given an LVR solution with an impermeant (gluconate, PEG-20k, or both) relative to the saline control group. Lactate in the BSA group after full resuscitation was significantly higher than all of the groups, including the saline controls.

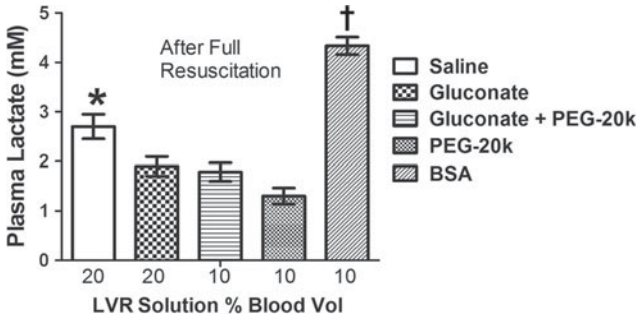

FIGURE 5. Plasma lactate values measured after 1 hour of full resuscitation in the 5 shocked rat groups. Values are mean ± SD, n = 6–12 animals per group, $^{*}\dagger P < 0.05$ relative to every other group.

Regional capillary blood flow in major organs and tissues in shocked rats treated with gluconate or with saline is shown in Figures 6 and 7. Local blood flow in the skeletal muscle, left ventricle, and brain (medulla) was significantly higher during the LVR period when an impermeant-based LVR solution was used, compared to saline. There were higher trends in other tissue beds too. After full resuscitation, regional blood flow was significantly higher in the left ventricle after impermeant-based resuscitation compared to saline. Again, there were strong trends in other beds.

ABG data are shown in the Table for rats given saline, saline with gluconate, or saline with gluconate + PEG-20k during the LVR period. ABG parameters are reported for each group after the baseline period before shock, after the hemorrhagic shock period, and after the LVR period (immediately before full resuscitation). In all groups, the changes in the ABG data from baseline to shock are predictable and not different between groups. Specifically, lactate rose to 10 mM in each group because the amount of shock that was induced was titrated and controlled to that level of oxygen debt (lactate). In addition,

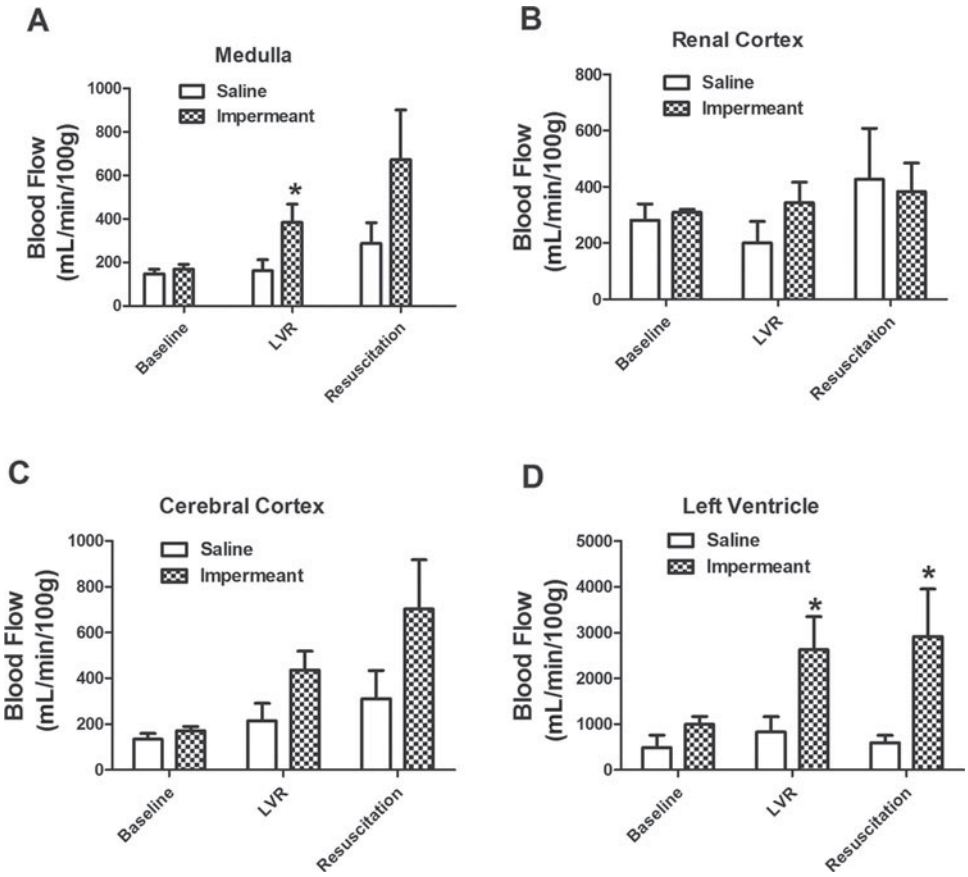

FIGURE 6. Capillary blood flow in medulla, cerebral cortex, renal cortex, and left ventricle at baseline, after the LVR period, and after the full-resuscitation period in 6 rats per group. The 2 groups compared were saline LVR solution (Saline) and a saline LVR solution with an impermeant mixture. $^{*}P < 0.05$ relative to the corresponding value for saline. Capillary blood flow was measured using the colored microsphere technique.

HCO_3^- and pCO_2 values fell as the pH remained unchanged. After LVR, however, some differences in the ABG data were apparent between the group receiving PEG-20k in the LVR solution and the other groups. Specifically, PEG-20k LVR prevented lactate levels from significantly rising above baseline (1.2 ± 0.4 mM during baseline vs 2.6 $\pm$ 1.1 mM after a 240-minute LVR period). A significant metabolic alkalosis with higher HCO_3^- and higher pH was also observed with PEG-20k resuscitation, relative to the other LVR groups. The pH of all of the LVR solutions was 7.2.

DISCUSSION

Severe hemorrhagic shock in the field can be life threatening because the blood pressure drops and the microcirculatory exchange capacity deteriorates, which cause the delivery of oxygen to tissues (DO_2) to fall. First responders are severely limited in what they can do to stabilize the DO_2. Recognizing now that high-volume crystalloid resuscitation that was once used to raise perfusion pressure is harmful, prehospital care now amounts to delivering LVR solutions. Given those constraints, LVR (<500 mL) should be looked upon as a vehicle to deliver agents that increase tolerance to the low-volume state rather than as a temporary volume expander to raise blood pressure per se. This is best accomplished by targeting significantly important causal mechanism and pathways of global ischemia and resuscitation injury. This study targeted cell swelling, which is a very specific and highly underestimated mechanism that contributes to the phenotypic changes associated with hemorrhagic shock and global ischemia.

Hemorrhagic shock is characterized by changes secondary to the loss or reduction in cellular energetics. As the cell ATP levels fall because of low oxygen delivery, the cell begins to lose ATP-dependent processes, including the active volume control mechanisms driven by the Na/K ATPase pump. Hydropic cellular degeneration then leads to cell and organelle membrane dysfunction, which can cause cell homeostasis abnormalities, lysis, and death. Furthermore, swollen parenchymal cells compress capillary exchange vessels to reduce capillary blood flow, which causes more ischemia and swelling in a vicious cycle. Although this mechanism is well appreciated in preservation injury of organs stored for transplantation, it is mysteriously unappreciated in global warm ischemia associated with shock, stroke, or infarction injury. The main objective of this study was to test this mechanism of shock by attempting to reverse it with cell impermeants that are known to prevent cell swelling but have few other biological effects. The results are clear, dramatic, and may represent a significant step forward in treating severe hemorrhagic shock with low-volume crystalloid-based resuscitation, especially in an austere prehospital environment.

Cell swelling plays a major role in organ preservation injury and may do the same in circulatory shock after trauma. Organ preservation causes cell swelling because depletion of ATP during cold ischemia and cold per se cause disruption of the normal ATP-dependent

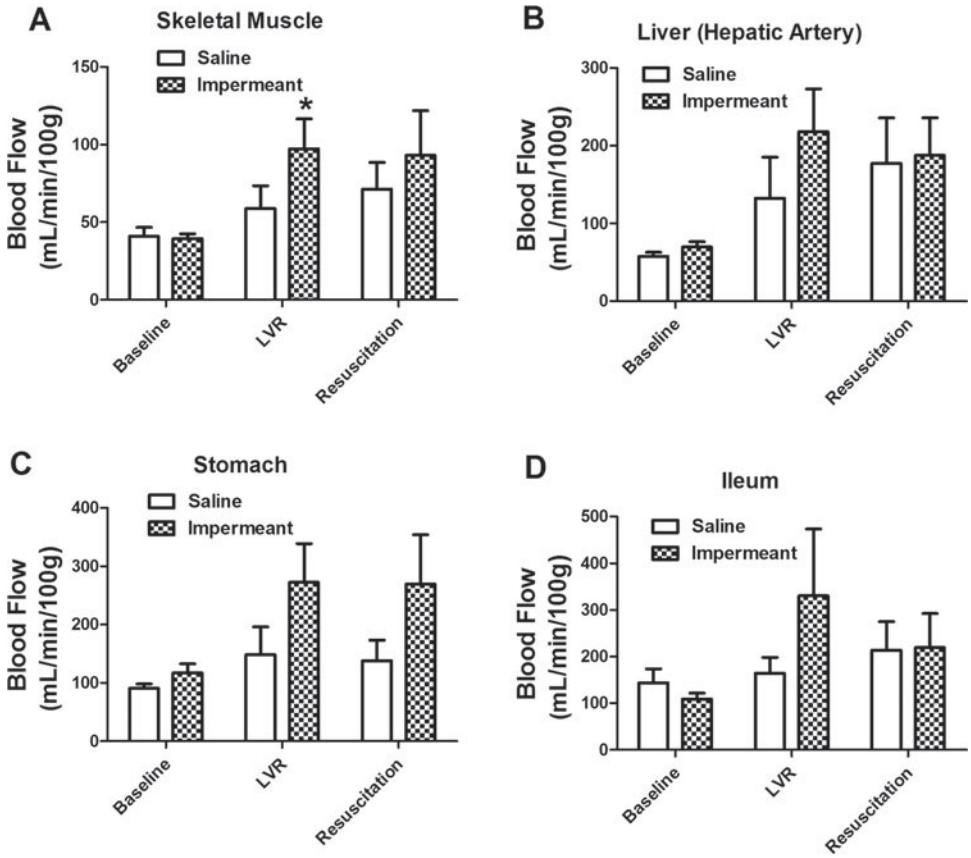

FIGURE 7. Capillary blood flow in skeletal muscle, stomach, liver (hepatic artery flow), and terminal ileum at baseline, after the LVR period, and after the full-resuscitation period in 6 rats per group. The 2 groups compared were saline LVR solution (Saline) and a saline LVR solution with an impermeant mixture. $^{*}P < 0.05$ relative to the corresponding value for saline. Capillary blood flow was measured using the colored microsphere technique.

cell volume control mechanisms. Cell swelling is a major contributor to preservation injury in recovered donor organs because it can be largely mitigated by using cell impermeants in organ preservation solutions. In fact, cell impermeants are one of the most important and effective components of modern day organ preservation solutions.[17] The concept is simple, load the interstitial space with molecules that escape the capillary but are impermeant to the cell membrane. This preferentially increases the extracellular osmolarity and prevents water from moving into the cell, which is its natural propensity when the intracellular sodium concentration increases after ischemia-induced pump failure. A similar mechanism is proposed to exist in ischemic shock and a similar solution was tried because most cell impermeants are relatively nontoxic and can be administered in high enough concentrations to be theoretically effective at preventing ischemic cell swelling. The evidence supporting this parallel mechanism is seen in the impermeant effects on liver cell swelling after warm ischemia and the effects of impermeants on LVR times, blood lactate levels after shock, and capillary blood flow to major organs during shock. As cell swelling is prevented with gluconate in the LVR solutions, microcirculatory exchange improves, which lowers plasma lactate values in treated subjects. This is manifest as both increasing LVR times and higher capillary blood flow to vital organs with impermeant treatment during the low-volume period. This is consistent with reduced swelling compression on microcirculatory exchange vessels and reduced obstructive swelling of endothelial cells forming the capillary lumen,[23–25] which allows for better capillary perfusion and more efficient cellular metabolism (lower lactates). All these data are consistent with the hypothesis that circulatory shock states promote cell swelling, which is an important cause of tissue, organ, and systems injury, acting in part, through a microvascular mechanism.

In an attempt to further test the cell-swelling hypothesis and to make impermeant treatment more effective, a model of a microcirculatory osmotic gradient was developed and tested. In the osmotic gradient model, 3 microvascular compartments are identified in a shocked patient as intracellular, interstitial, and the capillary compartment. An osmotic gradient could be established both between the intracellular and extracellular space by the use of conventional cell impermeants (like gluconate), which occupy both capillary and interstitial spaces, and a gradient could be established between the interstitial and capillary spaces by the addition of an oncotic agent to the circulation, which only occupies the capillary space. The combination of impermeant and oncotic agent would create this double gradient, which may not only prevent cell swelling but also keep water flowing out of the interstitial space and into the capillary space where it belongs. There, the water that would otherwise have entered the ischemic cells in the tissue will expand the circulatory volume and promote capillary blood flow and oxygen exchange, which mitigates the shock state by increasing the efficiency of oxygen delivery during low flow. When this was tried by combining both gluconate (an impermeant) with PEG-20k, an oncotic agent, a *huge* potentiation

TABLE. Blood Gas Data During the Shock and LVR Protocol in Rats Receiving Saline, Gluconate (Glu), or Gluconate + PEG-20k LVR Solutions

Group	ABG Parameter	Baseline	Hemorrhagic Shock	LVR
Saline (20% BW)	Lactate (mM)	1.35 (0.23)	9.72 (0.83)	9.49 (1.95)
	HCO_3^- (mM)	24.1 (2.6)	14.5 (3.17)	14.2 (1.63)
	pO_2 (mm Hg)	441 (32)	381 (63.8)	369 (36.3)
	PH	7.37 (0.04)	7.37 (0.04)	7.36 (0.05)
	pCO_2 (mm Hg)	39.8 (3.10)	26.4 (3.75)	22.9 (3.32)
Glu (20% BW)	Lactate (mM)	1.15 (0.33)	9.34 (0.37)	10.5 (1.67)
	HCO_3^- (mM)	26.2 (1.47)	16.3 (1.55)	13.0 (2.92)
	pO_2 (mm Hg)	406 (49.1)	398 (38.1)	379 (62.6)
	PH	7.39 (0.05)	7.39 (0.05)	7.30 (0.15)
	pCO_2 (mm Hg)	41.6 (3.11)	26.1 (2.59)	22.1 (2.76)
Glu + PEG-20k (10% BW)	Lactate (mM)	1.20 (0.37)	9.42 (0.21)	2.62 (1.06)*
	HCO_3^- (mM)	25.8 (1.28)	16.3 (0.99)	31.2 (2.54)*
	pO_2 (mm Hg)	448 (39.7)	417 (19.9)	419 (31.4)
	PH	7.40 (0.03)	7.34 (0.03)	7.48 (0.02)*
	pCO_2 (mm Hg)	36.1 (2.95)	25.2 (1.94)	39.9 (5.67)*

Values are mean ± SD, n = 6 per group.
*$P < 0.05$ relative to the corresponding value in the other LVR groups.
BW indicates volume based on calculated body weight.

effect was seen on LVR times. Furthermore, blood pressure during the LVR period was completely normalized with PEG-20k LVR solutions. Although gluconate doubled the LVR time, relative to the saline control, gluconate and PEG-20k increased the LVR time five- to six-fold. It is not even known what the upper limits are to this effect since the PEG-Gluconate studies were cutoff by the experimenter 5 hours after the start of the LVR period. At that time, the lactate levels were still only at about 2.5 mM, far below the threshold of 10 mM needed to trigger full resuscitation in our model. Furthermore, the volume of PEG-20k or gluconate needed for this effect was half of the volume used for the saline control LVR group. This supports the concept that the addition of PEG-20k served to move water into the capillary space where it supports intravascular volume, blood pressure, and microcirculatory flow. The latter is supported by the low plasma lactate levels throughout the LVR period, which suggest good microcirculatory flow secondary to high capillary driving pressures (fluid expansion). What this means clinically is that a severely shocked patient (MAP in the 40s with 50% blood loss) can receive half of the volume of an impermeant-based LVR solution (intravenously) and safely remain in the low-volume state for at least 6 times longer than if conventional saline resuscitation were used, before definitive full resuscitation is needed.

LVR solutions containing PEG-20k largely prevent the accumulation of lactate in the blood even after 240 minutes after the initial 55% blood volume hemorrhage. Accompanying the low lactate was a slightly higher pH and a significantly higher bicarbonate concentration (double that of the other LVR groups). This metabolic alkalosis served to correct the lactacidosis of the low-volume state and is likely of renal origin since the pCO_2 remained normal and the pH of the LVR solutions were held at 7.2. After administration of the LVR solution in these studies, we always observed a temporary diuresis, which was attributable to the osmotic retention of water in the renal tubules secondary to PEG-20k filtration across Bowman's space and trapping in the tubular lumen. This diuresis (and maybe a concomitant natriuresis) may result in the significant excretion of hydrogen ions into the urine resulting in a normalization of pH and even a slight alkalosis. Metabolic studies are needed to define this possible mechanism. In any case, preserving proper pH during shock and LVR may help maintain the normal blood pressure observed in the PEG-20k LVR group.

To test the oncotic-impermeant model further, we conducted studies using albumin and PEG-20k alone in the LVR solution. Because high-molecular-weight PEG molecules are known to have other biological properties besides their oncotic ones, we used the physiological prototype oncotic agent albumin as an oncotic control. Albumin used alone to control for oncotic effects was not at all as effective as PEG-20k alone but it was better than saline. This suggests that there is something different about PEG-20k. The effects of PEG-20k in LVR shock models are attributable to more than just purely oncotic properties. There are 2 reasonable possibilities to consider: (1) The involvement of nononcotic PEG effects such as PEG's known effects on cell membranes, protein binding and hydration properties, or immunocamouflage effects or (2) Oncotic-impermeant hybrid effects, where, because of the unique molecular weight and attributes of PEG-20k, it is able to act both as a cell impermeant and as an oncotic agent.

There is evidence to support the idea that PEG-20k acts both as a cell impermeant by escaping the capillary space while remaining impermeable to the cell and as an oncotic agent whereby a large amount of the material remains trapped in the capillary space. This property may be caused by a slow equilibration time to cross the capillary barrier into the interstitial space, based on its size and other attributes. In fact, PEG-20k has been shown to effectively expand the vascular space and move water out of the interstitial space to stimulate thirst in rats and pigeons,[26] which demonstrates its oncotic effects. It has also been detected immunohistochemically in renal tubule epithelium and in monocytes in the liver and lung after intravenous administration, suggesting that it leaves glomerular capillaries and hepatic sinusoids,[27] which demonstrates its partial impermeant effects. Our own studies and observations indicate that it leaves Bowman's space because a significant but temporary diuresis is seen in rats after severe shock after receiving PEG-20k in the LVR solution. This diuresis may have been due to PEG-20k-induced restoration of the arterial pressure during the LVR period or it may have been due to an osmotic diuresis from PEG being filtered and trapped in the renal tubules, similar to mannitol. The latter is more plausible because we do not see a diuresis in shocked rats when their blood pressure is normalized using conventional resuscitation solutions but we do with PEG-20k solutions. Normalization of renal perfusion pressure and the institution of a mild filtration may be desirable during a shock state, as long as the diuresis does not jeopardize the newly normalized blood pressure. There is no evidence in our studies that this happens. The renal effects would tend to prevent the development of acute tubular necrosis after resuscitation. The diuresis observed in

this study with PEG-20k is temporary and dose dependent because a 10% blood volume LVR dose of PEG-20k produces much milder diuresis compared to a 20% blood volume LVR dose. That's why we decreased the dose of the LVR solutions containing PEG-20k from 20% to 10%. The molecular weight of PEG-20k seems to be right on the size limit for partial capillary permeability because higher molecular weights approaching 30 kDa do not cross capillary spaces including Bowman's space.[27] A proposed hybrid oncotic-impermeant property of PEG-20k is also consistent with the observation from our study that PEG-20k was as effective alone as it was in combination with gluconate. In essence, partial capillary permeability characteristics of PEG-20k may allow enough osmotically active material to escape into the interstitial space to mimic the impermeant effect of gluconate while the majority of material stays behind in the capillary to act oncotically. Therefore, gluconate or other impermeants may not be necessary in LVR solutions using just PEG-20k alone, but further testing in survival studies is needed.

There are limitations to this study and to its projected clinical use. Our study used a controlled hemorrhagic shock model, which is highly relevant in limb or extremity injuries in the field or other compressible hemorrhagic injuries. In both cases, good hemorrhage control can be achieved with tourniquets and compression techniques. This stops the bleeding and allows the impermeant-based LVR solutions to expand the circulatory volume, drive up arterial pressure, and improve flow and oxygen exchange (in addition to protecting tissues from lethal cell swelling). In trauma cases, where bleeding remains uncontrolled or hard to control, the model is oversimplistic and overestimates its clinical utility because bleeding will continue. Bleeding will continue because (1) compression or hemostasis in the field is limited, (2) the increased arterial pressure (in the absence of hemostasis) from the osmotic volume shifts will exacerbate the pressure gradient for further hemorrhaging, and (3) the crystalloid solution does not provide any replacement of clotting factors and precursors, which could limit bleeding by active coagulation and platelet activation. These factors all limit the use of an impermeant-based LVR solution in many clinical settings. However, combining impermeant-based LVR solutions with plasma product replacements such as fresh frozen plasma or spray-dried plasma products may prove the most useful in many prehospital trauma settings involving uncontrolled hemorrhaging.

CONCLUSIONS

Cell swelling due to global ischemia from severe hemorrhagic shock plays a significant role in the sensitivity of the victim to the low-volume state. This is attributable to effects on the microcirculation with improved efficiency of microvascular oxygenation and perfusion during low-volume states. The use of cell impermeants with oncotic agents or PEG-20k alone in LVR solutions dramatically increases the time that a patient can safely remain in the low-volume state until definitive medical care and full resuscitation are needed. The new LVR solution may be important in civilian prehospital resuscitation and for combat casualty care and resuscitation on the battlefield, especially when combined with plasma component replacement.

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D.P. and S.L.L. contributed equally as first authors.

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New low-volume resuscitation solutions containing PEG-20k

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BACKGROUND: Hypovolemic shock reduces oxygen delivery and compromises energy-dependent cell volume control. Consequent cell swelling compromises microcirculatory flow, which reduces oxygen exchange further. The importance of this mechanism is highlighted by the effectiveness of cell impermeants in low-volume resuscitation (LVR) solutions in acute studies. The objectives of this study were to assess impermeants in survival models and to compare them with commonly used crystalloid solutions.

METHODS: Adult rats were hemorrhaged to a pressure of 30 mm Hg to 35 mm Hg, held there until the plasma lactate reached 10 mM, and given an LVR solution (5–10% blood volume) with saline alone (control) and saline with various concentrations of polyethylene glycol-20k (PEG-20k), Hextend, or albumin. When lactate again reached 10 mM following LVR, full resuscitation was started with crystalloid and red blood cells. Rats were either euthanized (acute) or allowed to recover (survival). The LVR time, which is the time from the start of the LVR solution until the start of full resuscitation, was measured as was survival and diagnostic laboratory values. In some studies, the capillary oncotic reflection coefficient was determined for PEG-20k to determine its relative impermeant and oncotic effects.

RESULTS: PEG-20k (10%) significantly increased LVR times relative to saline (eightfold), Hextend, and albumin. Lower amounts of PEG-20k (5%) were also effective but less so than 10% doses. PEG-20k maintained normal arterial pressure during the low-volume state. Survival of a 180-minute LVR time challenge was 0% in saline controls and 100% in rats given PEG-20k as the LVR solution. Surviving rats had normal laboratory values 24 hours later. PEG-20k had an oncotic reflection coefficient of 0.65, which indicates that the molecule is a hybrid cell impermeant with significant oncotic properties.

CONCLUSION: PEG-20k–based LVR solutions are highly effective for inducing tolerance to the low-volume state and for improving survival. (*J Trauma Acute Care Surg*. 2015;79: 22–29.

KEY WORDS: Cell swelling; shock; polyethylene glycol; rats.

Deaths caused by injury in the United States reached more than 190,000 and cost more than $400 billion a year in health care costs and lost productivity in 2012.[1] Deaths from trauma are the number one cause of death for people younger than 44 years in the United States and the third leading cause of death overall for all age groups. Trauma accounts for approximately 30% of all life-years lost in the United States, compared with cancer (16%), heart disease (12%), and human immunodeficiency virus (2%).[2] For all traumatic injuries, hemorrhagic shock is responsible for more than 35% of prehospital deaths and more than 40% of all deaths within the first 24 hours. This is second only to trauma deaths induced by severe central nervous system injury.[3] Finally, hemorrhagic hypotension exposes the patient to immediate complications of life-threatening infections, coagulopathies, and multiple-organ failure.[4,5]

Early resuscitation strategies include the use of low volumes of intravenous blood products to increase oxygen delivery and to replace lost coagulation and clotting factors (coagulation proteins and platelets). While this approach is fine for hospital emergency departments, it is not currently practical in prehospital settings where early intervention may be the key to preventing future complications following more definitive resuscitation. Crystalloids are available for prehospital use because they can be safely transported and stored but they are generally limited in their effectiveness. Attempts to modify basic intravenous crystalloids for prehospital resuscitation by adding hypertonic NaCl or starch (Hextend) as a volume expander have had disappointing results.[6,7] The future use of effective spray dried blood products will be a valuable tool in prehospital settings since they replace chemical coagulation precursors and factors. The use of fresh frozen plasma in the field, which is currently being tested at many centers, will also be useful, but it too is limited by the need for refrigeration.[8] There remains a need for a better crystalloid to resuscitate patients with severe hemorrhagic shock, especially in a prehospital setting. The successful design of such a solution is highly dependent on understanding the pathophysiologic mechanisms that lead to injury during hemorrhagic hypotension and subsequent resuscitation. The optimal solution will likely be an effective new stable crystalloid that targets these mechanisms used together with reconstituted dried plasma products for the replacement and reconstitution of coagulation potential.

The predominant root mechanism of injury in hemorrhagic shock is energy failure. While global ischemia and reperfusion injury are causally based at many levels, they all arise

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from changes that occur when the cell energetics drops because of a loss of adequate microvascular oxygen transport and subsequent loss of aerobically produced high-energy adenine nucleotides.[9–11] One mechanism of cell, tissue, and organ injury is cell swelling that occurs from the loss of adenosine triphosphate (ATP)–dependent cell volume regulatory control mechanisms. In most cells, the single highest energy consuming process is the running of the Na/K ATPase pumps in the cell membrane. These pumps actively transport sodium ions out of the cell to maintain membrane potentials and to run numerous Na+-dependent facilitated membrane transport processes such as calcium, glucose, amino acids, and organic cation transporters. In the absence of ATP to run those pumps, as occurs in ischemia following hemorrhagic shock, the Na/K ATPase turns off, and sodium enters the cell as it runs back down its electrochemical gradient. The elevated intracellular sodium futilely stimulates the sodium pump that cannot run because of the loss of ATP.[12] Chloride then enters the cell down an electrical gradient, and water follows the sodium chloride down a developing osmotic gradient, which causes the cell to swell. Hydropic degeneration from energy failure damages membrane and mitochondrial structures,[13] which may lead to cell death. Swelling of parenchymal cells can also compress local capillaries, leading to further reduced capillary flow and oxygen delivery causing a self-amplifying cycle. Figure 1 shows how this mechanism occurs and how novel cell impermeant molecules can passively reverse this dangerous water flow.

This basic mechanism of cell ischemic injury has been well described in organ preservation associated with transplantation.[14–16] Effective modern organ preservation solutions were developed around this concept and contain high concentrations of cell impermeants.[17] These are classes of nontoxic molecules, usually saccharides and small organic cations and anions, which are small enough to freely egress the capillary space in the

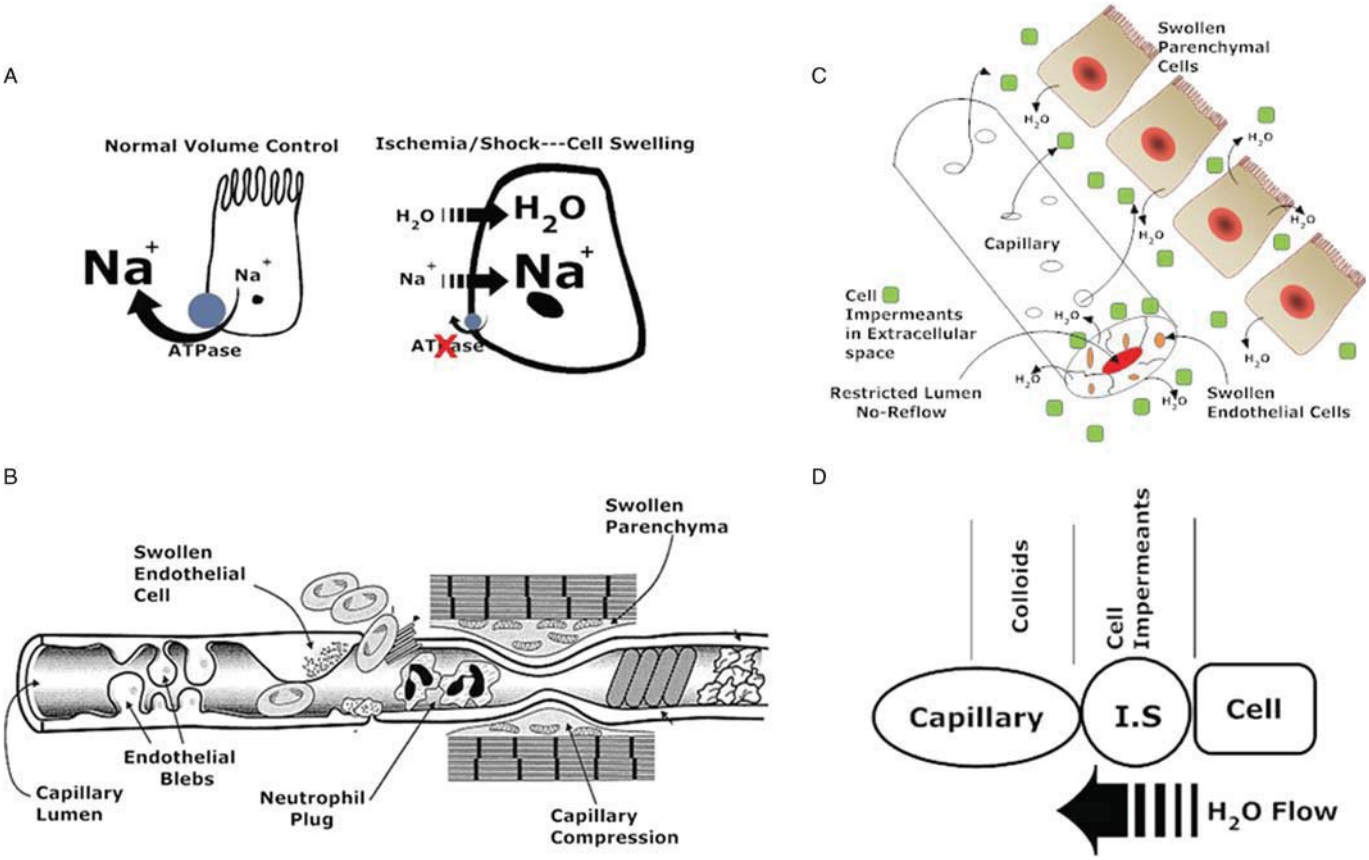

Figure 1. Proposed mechanism of action of cell impermeants in the nonenergetic rebalancing of water movements during low-volume shock states. *A*, The original defect is caused by the energy-dependent collapse of the Na/K ATPase activity during shock because of low oxygen delivery and loss of ATP. As the pump fails, Na^+ enters the cell followed by water. *B*, Swollen parenchymal cells compress local capillary networks in the tissue that increase the resistance to capillary blood flow and further impede microcirculatory oxygen delivery. This allows local lactates to rise. *C*, Loading the interstitial space with cell impermeants such as gluconate or raffinose prevents ischemia-induced water movement (swelling) by osmotically holding water in the interstitial space. This prevents capillary compression and preserves local exchange capacity, even under low-volume conditions. *D*, The inclusion of an oncotic molecule with an impermeant establishes an osmotic-oncotic gradient between the intracellular-interstitial-capillary compartments, which promotes further the energy independent flow of water from the cell (where it should not be) into the capillary (where it should be). The movement of capillary water with oncotic agents then increases capillary pressures that promote capillary flow even under low-volume states. The sum effect is to promote effective and efficient capillary transport and oxygen delivery in the low-volume state.

microcirculation but are too large or too charged to cross the cell membrane. As such, they preferentially load into the interstitial space where they create an osmotic force that prevents the movement of water into the cell as the sodium concentrations rise during ischemia. They prevent lethal cell swelling. Cell impermeant, as a class of agents, are one of the most effective components of organ preservation solutions used today.[18] The University of Wisconsin solution contains high amounts of raffinose, lactobionic acid, sulfate, and phosphate, which all act as cell impermeants to prevent water movement. The Belzer-UW MPS solution uses gluconate, and HTK solution uses both high concentrations of histidine and mannitol as impermeants. Water movement in organ preservation is slower than ischemia at normal mammalian temperatures because hypothermia is used to preserve organs, which slows down the process. Since cell swelling during ischemia induced by hemorrhagic hypotension also occurs[19] and at a much faster rate than in organ preservation because of the warmer temperatures, it was hypothesized that loading the interstitial space with nontoxic cell impermeants during the low-volume period would prevent lethal cell swelling and increase the tolerance of the patient to the low-volume state and improve outcomes at resuscitation. In fact, acute studies in rodents with severe hemorrhagic shock indicate that small cell impermeants double the tolerance of animals to the low-volume state.[20] This study further found that one particular molecule, polyethylene glycol-20k (PEG-20k) increased the tolerance to the low-volume state 6 fold, compared with saline controls. It was hypothesized that PEG-20k superiority was due to the molecule behaving as hybrid where some escapes the capillary space to act as an impermeant to prevent water movement into the cell and a large portion of the molecule stays behind in the capillary to exert oncotic force that draws the interstitial water into the capillary. This was supported by observations that PEG-20k in LVR solutions also normalizes the arterial blood pressure in the low-volume state immediately after administration.[20] This previous study neither assessed the effects of PEG-20k based LVR solutions in a survival model nor compared them with standard crystalloid solutions used today. This was the objective of the current study. We hypothesize that PEG-20k added to LVR solutions possesses both impermeant and colloidal properties that greatly improve outcomes in a low-volume resuscitation (LVR) model of severe hemorrhagic shock.

MATERIALS AND METHODS

All animal work was conducted under a protocol approved by the VCU Institutional Animal Care and Use Committee, which is governed by the rules and regulations set forth in the National Institutes of Health guide and the US Department of Agriculture.

Rodent Shock Model

An LVR model was used in adult rats to test the impermeant-based LVR solution used for prehospital resuscitation of rats with severe hemorrhagic shock. Adult Sprague-Dawley rats were anesthetized with isofluorane and maintained in a light surgical plane of anesthesia during the study. Polyethylene catheters were placed in both femoral arteries for blood pressure monitoring and blood sampling, and a catheter was placed in one femoral vein for administration of fluids. The animals were allowed to ventilate on their own to establish normal arterial blood gas (ABG) values. A 1-cm midline incision was created to induce soft tissue injury and for the placement of a temperature probe in the abdomen. The animals were kept at 38°C using a heating pad and an incandescent light source above them. Arterial blood pressure, heart rate, and temperature were continuously recorded using a PowerLab (ADInstruments, Boston, MA). After a 30-minute stabilization period, heparin was given (500 U/kg), and arterial blood was slowly removed at 1 mL/min into a syringe to maintain blood pressure at 30 mmHg to 35 mm Hg. This hypotension was maintained until the plasma lactate reached a value between 9 mM and 10 mM, as measured every 15 minutes with a handheld lactate analyzer (Lactate Plus, Nova Biomedical, Waltham, MA) and every hour with a blood gas analyzer (Radiometer 800). In preliminary studies, 9 mM to 10 mM was the highest plasma lactate level achievable without mortality during the LVR period. Once the target lactate was reached, an LVR equal to 5% to 10% of the calculated blood volume[21] of saline was administered intravenously over a 10-minute period using a syringe infusion pump. When the blood lactate again reached 9 mM to 10 mM, full resuscitation was started, which consisted of a volume of saline equal to the volume of the blood loss (approximately 55–60% of total blood volume) plus 30% of the removed red blood cells (washed) infused intravenously over 10 minutes. After 1 hour of full resuscitation, the animals were euthanized by an anesthetic overdose, and terminal blood was removed for analysis. The time from the start of the LVR period until the start of full resuscitation is called the LVR time, and it represents the tolerance of the animal to the low-volume state or the maximum amount of time that a shocked subject can safely remain in the low-volume state until more definitive resuscitation is required. This was a major outcome used in the study. In some experiments, survival from severe shock was studied with impermeant-based LVR and compared with saline controls. In these studies, the animals were held in the low-volume state for 180 minutes receiving either 10% saline as a control or 10% saline containing 10% PEG-20k impermeant. After 180 minutes, the animals were given full resuscitation and were awaken from anesthesia after the catheters were removed. These surviving subjects were studied the following day (24 hours) to determine the rate of survival, blood pressure, lactates, base excess, Pa_{O_2} (A-a gradient), and other blood laboratory values. The shock and LVR protocol is illustrated in Figure 2.

Oncotic Reflection Coefficient

The oncotic reflection coefficient (σ_d) of PEG-20k in rodent capillaries was determined to characterize the biophysical characteristics of this impermeant in capillary networks. The σ_d describes the relative convective solvent drag transport of a molecule across capillary pores. This characteristic is diffusion independent and is measured by determining the ratio of a compounds lymph concentration to the plasma concentration at high lymph flow rates. In these studies, rats were anesthetized as before, and a PE10 cannula was introduced into the thoracic duct as previously described[22] to direct the lymphatic flow into a collection tube. Heavy cream (5 mL) was injected into the stomach to help visualize the duct after the lipid was absorbed. A

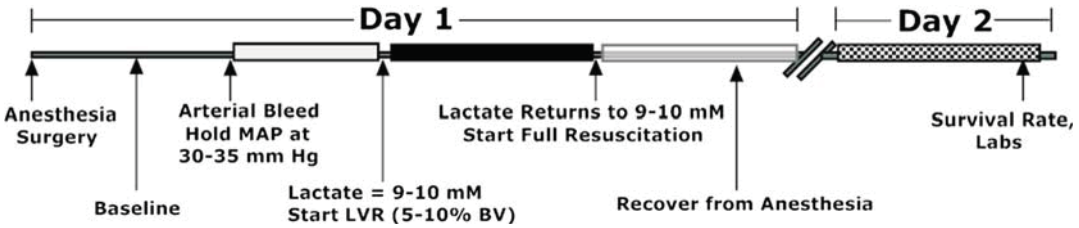

Figure 2. Diagram showing the shock, resuscitation, and recovery protocol used for these studies. The LVR time is the time from the start of the LVR (after the lactate during hemorrhagic shock reaches 9–10 mM) until the time after the LVR infusion when the lactate rises back up to 9 mM to 10 mM again. This immediately precedes full resuscitation. The LVR time is a measure of the tolerance to the low-volume state and is a function of the microcirculatory effectiveness since it is dependent on the rate of change of the plasma lactate.

saline infusion (intravenous) was started at 0.25 mL/min to accelerate lymph flow. Then, a single-bolus injection of 5-mg FITC-labeled PEG-20k (Nanocs, New York, NY) in saline was given. Blood plasma and lymphatics were collected every 10 minutes for an hour. FITC-PEG was then quantitated by direct measurement of the FITC fluor using a fluorescence plate reader (Biotek FL-800) with an excitation wavelength at 485 nM and an emission wavelength at 520 nM. The σ_d was estimated to be 1-L/P of FITC-PEG-20k as previously described.[23] The values for σ_d are from 0 to 1 where 0 means no reflection into the capillary or complete freedom of passage through the capillary pores (impermeant characteristics). A σ_d of 1.0 means total reflection back into the capillary or complete oncotic properties.

Statistical evaluations of mean values were performed using parametric analysis of variance with multiple comparisons corrections using the Dunnett or Bonferroni test for more than two groups or an unpaired *t* test for comparison of data with only two values (control and test groups). Fisher's exact test was used for survival testing, and a *p* value of 0.05 was set as a cutoff for statistical significance.

RESULTS

The effects of LVR solutions on the LVR time are shown in Figure 3*A*. The LVR time in this model is an index measuring the tolerance of the individual to the low-volume state. It is the length of time that a patient can safely remain in the low-volume state until definitive medical care and resuscitation is needed (golden hour), as indexed by the accumulation of a critical level of oxygen debt (lactate). With the use of normal saline (10% blood volume) as a control base crystalloid, the LVR time was determined to be approximately 30 minutes. Specifically, the time from the start of the low-volume infusion (triggered when the patient accumulated a lactate of 10 mM) until the time when the patient reaccumulated the same lactate level was determined to be an average of 30 minutes. This was significantly increased to 240 minutes (eightfold increase) when the same volume of saline contained 10% PEG-20k. Compared with PEG-20k, the LVR times for traditional resuscitation solutions such as 10% Hextend or 10% albumin were significantly lower. The plasma lactate at the end of the LVR period was close to 10 mM in all groups because this level of oxygen debt triggered the end of the LVR period by definition. However, the plasma lactate in the PEG-20k group at the end of 240 minutes was only 1.2 mM, which is far lower than the 10 mM trigger. Thus, the LVR time in the PEG-20k group (10%) was arbitrarily cut off and is a significant underestimation of its true value.

During the low-volume state after the LVR solution is administered, mean arterial blood pressure (MAP) was measured for the duration of the LVR period for a variety of LVR

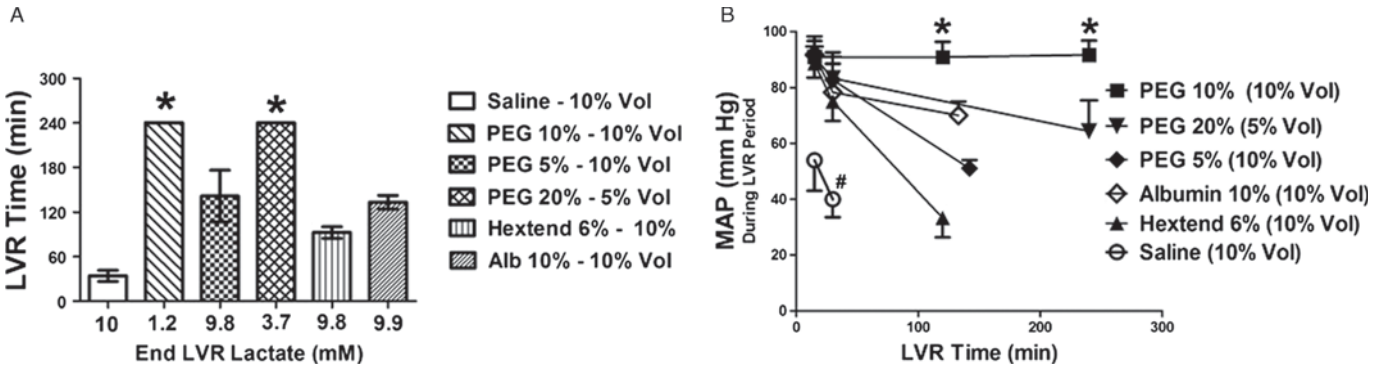

Figure 3. *A*, LVR times for rodents in acute studies comparing the effectiveness of LVR solutions containing PEG-20k, Hextend, albumin, and saline controls. The numbers on the x-axis are the corresponding lactate concentrations in the plasma at the end of the LVR period. Most are close to 10 mM because that was the definition of the end of the LVR period. All agents were at a concentration of 5% to 20% by weight and were delivered at a volume equal to 10% or 5% of the calculated blood volume. *$p < 0.05$ relative to all other values, all treated groups are significantly different from saline (control), all values are mean (SD), n = 6 to 10 per group. *B*, MAP measured at 15 minutes, 30 minutes, and at the end of the LVR period for (6) groups of rats treated with various amounts of PEG-20k, albumin, Hextend, or saline as the LVR solution. *$p < 0.05$ relative to the other corresponding groups, #$p < 0.05$ relative to all other corresponding values, n = 6 to 10 per group, all values are mean (SD).

TABLE 1. The Effects of PEG-20k LVR Solution on Hemorrhagic Shock Values During the LVR Period and After Full Resuscitation in Survival Animals

During LVR

	LVR Time, min	MAP, mm Hg	Lactate, mM	HCO_3^-, mM	Pao_2, mm Hg
Saline (10%)	34 (18)	49.3 (11)	9.53 (2.1)	11.9 (2.1)	389 (72)
PEG-20k (10%)	180 (0)*	95.0 (3.5)*	1.42 (0.6)*	25.3 (3.4)*	465 (31)
Next-day survival					
	Survival, %	MAP, mm Hg	Lactate, mM	HCO_3^-, mM	Pao_2, mm Hg
Saline (10%)	0 (0)	NA	NA	NA	NA
PEG-20k (10%)	100 (0)*	85.6 (6.2)*	1.2 (0.1)*	25.6 (2.6)*	475 (80)*

*$p < 0.05$.
Values are mean (SD). Relative to corresponding saline values, n = 5, Pao_2 measured with an FIO_2 of 0.9.

solutions (Fig. 3*B*). Saline solution (10%) resulted in low MAP values during the 30-minute LVR time with values less than 60 mmHg and 40 mmHg at 15 minutes and 30 minutes, respectively. These pressures were improved with Hextend and albumin and completely normalized with PEG-20k (90–100 mm Hg).

Survival studies were conducted in a series of animals to determine the long-term survival effects of impermeant-based LVR solutions after a severe blood loss (55–60%) and a high accumulation of oxygen debt (lactate 10 mM). These results are shown in the Table 1. All animals were required to undergo a controlled 180-minute LVR time with a 10% LVR solution following the hemorrhagic shock protocol (Fig. 2). When saline was used, 0% survived for 24 hours, and most died within 30 minutes to 45 minutes after the LVR solution was administered. By comparison with the control, survival was 100% with the same volume of saline containing 10% PEG-20k. Furthermore, all of the animals that survived 24 hours after full resuscitation had normal blood pressure and ABG values, both during the 180-minute LVR period and after 24 hours of recovery. Saline-treated animals had very low pressures during the LVR period, they demonstrated aberrant ABG values that were characteristic of severe metabolic acidosis, and they did not report 24-hour values since they all died during the LVR period.

The capillary oncotic reflection coefficient for PEG-20k was measured in rats (Fig. 4). The reflection coefficient was determined to be approximately 0.65, which indicates that some of the fluorescently labeled PEG-20k marker was pushed across the capillary into the interstitial space and lymphatics while much of the label was also clearly detected in the capillary space (plasma). The oncotic reflection coefficients were measured under high lymphatic flow rates by administering an intravenous infusion of saline during the 1-hour study. These conditions unmask the convective solvent drag transport potential of the tracer rather than the diffusional transport characteristics.[24]

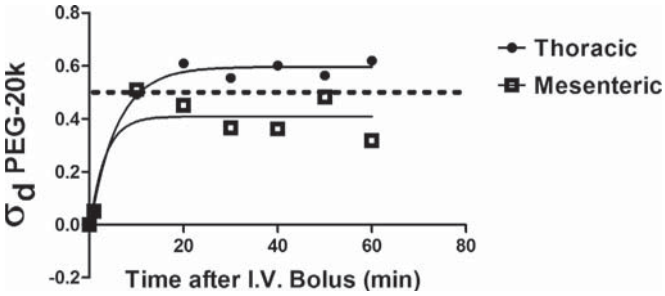

Figure 4. The oncotic reflection coefficient (σ_d) for PEG-20k was measured in six rats to determine the impermeant and the oncotic effects of this molecule in both the mesenteric vascular bed and the thoracic bed. FITC-labeled PEG-20k was used as a tracer molecule, and the reflection coefficient was determined by measuring the lymph (L) to plasma (P) concentrations of FITC-PEG after an intravenous injection of the tracer under conditions of high lymph flow induced by volume loading with intravenously administered saline infusions (0.25 mL/min). Lymph was sampled from a cannula placed into the thoracic duct to drain either the thoraces or the mesentery. FITC-labeled PEG-20k was measured by excitation-emission spectrofluorometry. The oncotic reflection coefficient was calculated as 1-L/P for PEG-20k at high lymph flow rates to make transport across the capillary totally dependent on convective solvent drag transfer and independent of diffusion. A coefficient of 1.0 indicates complete reflection back into the capillary and describes a pure oncotic agent. A reflection coefficient of 0 indicates no reflection at high lymph flow rates and describes a pure impermeant molecule (provided it is impermeant to cell membranes). The actual measured σ_d for PEG-20k was approximately 0.60 in the thoracic tissues and 0.40 in the mesenteric tissues, which suggest that the molecule is in fact behaving as a hybrid where some escapes into the interstitial space to act as an impermeant and a large amount of the material stays in the capillary where it behaves as an oncotic agent. The lower values in the mesentery is consistent with the known fenestrated ''leaky'' capillaries in the gut. The hybrid behavior is consistent with its physiologic effects on blood pressure and low-volume tolerance following shock. This property also explains why PEG-20k is effective by itself without classic impermeants (such as gluconate) added with it. The values of σ_d at each time point represent the average of three independent values from three animals for each vascular bed.

DISCUSSION

Prehospital resuscitation of patients in the field with severe trauma and hypovolemic shock is challenging since the first responders are often forced to work with low volumes of simple crystalloid solutions that are both transportable and

stable under field conditions. A recently described advancement in this area uses high concentrations of cell impermeant molecules in saline solutions such as LVR solutions for prehospital management of severely hypovolemic shock patients. In acute studies, these solutions prevent ischemia-induced cell swelling, which alleviates both the harmful effects on cell and mitochondrial membranes and greatly improves microcirculatory capillary flow and exchange by preventing the occlusion of the microcirculation by swollen parenchymal and endothelial cells. This study extends those findings by testing the effects in survival shock models and compares these effects to crystalloid solutions that are considered standard of care in the field today. Finally, this study explores further the unique mechanism of action of PEG-20k, which has been determined to be the most superior impermeant molecule yet tested for LVR in severe hypovolemic shock.

Cell impermeants are useful in severe shock because they load the interstitial space with osmotically active molecules that are impermeant to the cell membrane but freely escape the capillary space. The increased osmotic force generated outside of the cell prevents intracellular water accumulation, cell swelling, and secondary capillary compression (Fig. 1). The addition of an oncotic agent to a cell impermeant solution was hypothesized to potentiate the effect of the impermeant alone by establishing a second oncotic gradient between the capillary space and the interstitial compartment, thereby augmenting the translocation of water accumulated in the interstitial space by the impermeants into the capillary space. This nonenergetic movement of water into the capillary raises capillary pressure and increases capillary perfusion by both reducing the resistance to capillary flow (by preventing diameter changes from compression) and by increasing the capillary pressure gradient for flow. To our surprise, the addition of the oncotically active impermeant, PEG-20k, to LVR solutions containing simple impermeants such as gluconate geometrically potentiated the impermeant effect. The total response of the two components was reproduced by the PEG-20k component alone and much less than a pure oncotic agent alone (albumin). This suggested that PEG-20k may have a hybrid effect where the molecule acts both as a capillary permeable cell impermeant and as a traditional oncotic agent. Therefore, this study focused solely on the PEG-20k molecule.

An LVR solution containing 10% by weight of PEG-20k (given at 10% blood volume) has been shown to be optimal in our shock models. This was the criterion standard to compare other solutions to size up their clinical potential. A 10% PEG solution given at 10% of the calculated blood volume (approximately 500 mL for an adult patient) produced the longest tolerance to the low-volume state as compared with 6% Hextend and 10% albumin solutions. Since clinical formulations of albumin are generally approximately half strength (6% by weight), the values observed in this study probably are overestimations of the effects observed clinically. Furthermore, the effect of the PEG-20k group has been significantly underestimated in this study since the LVR times were cut off at only 240 minutes, which is lower than the true LVR time because the trigger of 10 mM was never achieved in this group. Had the LVR time been increased until the lactate in the PEG-20k group reached 10 mM, the final LVR time would have been much greater than 240 minutes. Therefore, the true limits of PEG-20k–based LVR solutions, regarding tolerance to the low-volume state, are not yet known.

In a dose deescalation trial, we compared 10% PEG-20k given at either a lower volume (5% blood volume) of the same concentration (10%) or a lower volume (5%) at twice the concentration (20%). The lower total PEG-20k dose was less effective, while the same dose but given at the lower volume was still very effective. This suggests that even lower volumes of LVR solutions can be achieved down to 5% of calculated blood volume. This is approximately 250 mL for an adult patient and may find use in combat casualty care on the battlefield where carry volumes of intravenous fluids for resuscitation are more of a concern.

The previous trials of impermeant-based LVR solutions in shock were acute studies. The effects on survival are an important consideration for possible clinical use. When the LVR time was controlled to 180 minutes, all of the animals resuscitated with 10% PEG-20k as the LVR solution survived 24 hours compared with 0% survival in the saline control group. Furthermore, the surviving rodents were perfectly normal both in terms of physiologic laboratory values and behaviorally. There were no apparent adverse effects of the PEG-20k LVR solutions except a temporary diuresis immediately after administration of the solution and a temporary metabolic alkalosis. Since it is believed that PEG-20k acts as a hybrid molecule, it is likely that some of the material passes across Bowman's space in the glomerulus where it acts as an impermeant in the tubules to increase osmotic water clearance and cause a diuresis, similar to a mannitol effect. Furthermore, the increased excretion of water and likely electrolytes too could prevent hydrogen ion reabsorption and increase renal acid excretion, thereby causing a metabolic alkalosis. This is a favorable effect in shocked patients that are experiencing severe metabolic lactacidosis and obviates the requirement of bicarbonate administration to correct acidosis during resuscitation.

The impermeant effect in LVR solutions is greatly augmented when a colloid is also present. Since the putative colloidal agent, PEG-20k, works as well by itself as it does with typical small-molecule impermeants such as gluconate, it was hypothesized that this size PEG polymer may act as a hybrid and possess both impermeant and colloidal properties. To support this hypothesis, the capillary oncotic reflection coefficient for PEG-20k was measured in rats (Fig. 4). The reflection coefficient was determined to be approximately 0.65, which clearly suggests that some of the material escapes into the interstitial space (impermeant characteristics) while a large portion of the material stays behind in the capillary to act oncotically. This strongly indicates a hybrid nature of PEG-20k, which supports the direct observations of its superior utility alone as an LVR solution and its apparent ability to cross the glomerulus to cause a diuresis. Further studies are needed to characterize the renal handling of PEG-20k, but its combined impermeant and colloid effects are now well supported.

There are three major limitations of the study. First, the model was for a controlled hemorrhage event and needs to be evaluated also in an uncontrolled model since many clinical conditions have an uncontrolled component. The current rodent model needs to be translated to a large animal preclinical study, and finally, the toxicologic effects of PEG-20k need more

evaluation, especially any possible platelet and coagulation responses (nothing has been identified to date in survival studies). Human trials are pending an Food and Drug Administration regulatory pathway decision.

In conclusion, PEG-20k used at 10% weight and administered at 10% calculated blood volume during severe hypovolemic shock produces striking salutary benefits. These effects dramatically prolong survival in a controlled hemorrhage model and are likely due to the molecules hybrid impermeant and oncotic properties.

AUTHORSHIP

Each author contributed significantly to and is willing to take public responsibility for one or more aspects of the study: its design, data acquisition, and analysis and interpretation of data.

DISCLOSURE

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EDITORIAL CRITIQUE

This manuscript produced by Drs. Parrish, Mangino and colleagues continue their investigations using cellular impermeants (in particular PEG-20k) in hemorrhagic shock resuscitation. Cellular impermeant such UW solution and PEG-20 K solutions attempt to reduce cellular edema and organ injury following shock by stabilizing water movement during resuscitation. By using both oncotic pressure and the cellular impermeant function of PEG solution the authors minimize organ edema and endothelial edema and reduce cellular ischemia and organ injury following hemorrhagic shock. Additionally, the authors' prior ex-vivo cellular work has validated a reduction in swelling in both ischemic hepatic and ischemic pulmonary cells treated with impermeants. In the current model, tissue water composition would be an important marker for the mechanism of action of this experimental intervention and the authors continued evaluation of in-vivo organ edema is encouraged.

The question regarding the impact of impermeant resuscitation on hemostasis is an important one. Prior cell impermeants such as UW solution have had significant problems with red blood cell aggregation in living models and as such have not been effective resuscitation fluids. Peg-20K does not appear to have these effects. Investigation into an uncontrolled hemorrhage model and further research into the effect on both platelet aggregation and the impact on fibrinolysis should be conducted to validate the author's impressive findings.

This novel resuscitation pathway could have significant impact on pre-hospital and acute resuscitation following injury by reducing the amount of fluid needed to be carried in the field by medical personnel and by allowing for low crystalloid volume resuscitation to adequately restore organ blood flow. The overall application of this resuscitation strategy is truly limitless. While the author's initial work is in hemorrhagic shock, patients suffer septic shock that require large volumes of crystalloid resuscitation could also benefit from this line of research. The authors should be commended on their continued research into this novel resuscitation method which has the potential for broad reaching impact on how we resuscitate patients from shock.

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Low Volume Resuscitation for Hemorrhagic Shock: Understanding the Mechanism of PEG-20k

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Running Head: Polyethylene Glycol Polymers in Shock

ABSTRACT

Hemorrhagic shock leads to cell and tissue swelling and no reflow from compressed capillaries. Cell impermeants, including polyethylene glycol-20,000 (PEG-20k), reverse ischemia-induced cell swelling, extend low volume resuscitation (LVR) time after shock, and increase tolerance to the low volume state. The purpose of this study was to explore the mechanisms of action of PEG-20k containing LVR solutions. We hypothesized that PEG-20k acts as both an oncotic agent and an impermeant in the microcirculation, which moves water out of the extracellular space and into the capillaries to affect peripheral capillary filling and enhanced perfusion during the low volume state. Rats were hemorrhaged until arterial lactate reached 9-10 mM/L. Then, saline-based LVR solutions containing various impermeant materials were administered (10% blood volume). The LVR times for these solutions was determined by measuring the amount of time required for plasma lactate to climb back to 9-10 mM after LVR administration (low volume tolerance). Capillary blood flow was measured by colored microspheres and blood volume was measured by FITC-labeled albumin dilution. Gluconate (impermeant), albumin (colloid), and PEG-20k (hybrid) increased LVR time over saline by 4-, 3-, and 8-fold, respectively. The combination of impermeant + albumin produced a biological effect that was similar to PEG-20k alone. Capillary blood flow and plasma volume was decreased after shock with saline LVR but increased with PEG-20k, relative to saline. These data are consistent with the hypothesis that PEG-20k may act by establishing multiple osmotic gradients in the microcirculation to drive cell-to-capillary water transfer during hypovolemic shock.

Key Words: Impermeant, Capillary Flow, Crystalloid

Introduction

Minimizing the use of crystalloids and utilizing blood products after trauma are now becoming mainstream in civilian trauma centers. Damage control resuscitation is also emerging as standard of care for the US Department of Defense, according to the Joint Theater Trauma Systems Clinical Practice Guidelines (JTTS CPG). When blood products are not available for resuscitation, crystalloid solutions are administered. However, only a fraction of infused crystalloid volume stays in the intravascular space and the use of low volume crystalloids has minimal effects on pressure and perfusion (1, 2). The movement of crystalloid fluid from capillary to interstitium is compounded by the increase in capillary permeability from trauma-related inflammation and trauma-induced capillary leak syndrome (TICS)(3). Furthermore, crystalloid resuscitation exacerbates TICS, acidosis, hypothermia, and coagulopathy (3, 4). Other resuscitation solutions such as hypertonic saline or starch have had disappointing results (5, 6) including concerns and risks associated with their use (4, 7). There remains a need for a better crystalloid fluid that can be given at a low volume to resuscitate patients in hemorrhagic shock awaiting definitive treatment, especially for the prehospital setting. A mechanistic approach was used to design such a solution.

The dominant mechanism of injury in hemorrhagic shock is energy failure secondary to lack of end-organ perfusion and loss of adequate microvascular oxygen transport with subsequent loss of aerobically produced adenosine triphosphate (ATP) (8). As cells lose ATP due to ischemia, the sodium pump shuts off and sodium ions enter the cell and accumulate as they run down their electrochemical gradient. Chloride follows electrogenically and water enters the cell osmotically. As water enters ischemic cells, they swell and compress nearby vascular

structures, which further aggravates ischemia by reducing local microcirculatory flow (9-11). Swollen vascular endothelial cells and parenchymal cells compress capillaries to cause no-reflow and promote resuscitation injury and limit oxygen delivery during the low flow state and after full resuscitation (10).

Cell swelling due to ischemia, hypothermia, and tissue energy failure has been best characterized in the organ preservation literature associated with transplantation, along with simple strategies to overcome it (12). Cell impermeants include sugars like sorbitol, gluconate, raffinose, lactobionate, and trehalose. All prevent cell swelling. Anions like sulfate, phosphate, and cations like histidine (at physiological pH) are also impermeants because they are small enough to escape the microcirculation but too large or too charged to enter the cell. Thus, they preferentially load into the interstitial space where they create a selective osmotic gradient opposing water movement into the cell. Modern organ preservation solutions like University of Wisconsin (UW) solution, among others, are largely effective because they contain high concentrations of cell impermeants (raffinose, lactobionic acid, gluconate, sulfate, and phosphate) (13). This organ preservation concept and strategy was recently developed for warm ischemia associated with shock in patients since these impermeant agents are both highly effective and relatively safe and nontoxic (1, 14, 15).

Parrish et al.(1) have demonstrated reduced ischemia-induced cell swelling, increased tolerance to the low volume state, and higher survival rates with administration of cell impermeant-based low volume resuscitation (LVR) solutions in a rodent model of severe hemorrhagic shock. It was reasoned that if this occurs because of the creation of an osmotic gradient for fluid movement during ischemia, then a second gradient created with the addition of an oncotic agent in the resuscitation solution would augment the response. Indeed, when

gluconate (a cell impermeant) was combined with polyethylene glycol 20,000 (PEG-20k, a colloid) in a low volume resuscitation crystalloid solution, a marked potentiation in low volume tolerance and blood pressure was observed (1). Surprisingly, when PEG-20k was used alone, it was equally effective as PEG-20k with the impermeant (gluconate). Additional studies demonstrated that PEG-20k, originally believed to be an oncotic agent, has both oncotic and impermeant effects because some of the material escapes the capillary space (impermeant effects) (15). This rather rare molecular behavior may explain how PEG-20k alone was orders of magnitude more effective at increasing shock tolerance compared to either saline, mixtures of cell impermeants alone, or pure oncotic agents alone (albumin). Specifically, this one agent may be doing double duty as both an oncotic and an impermeant molecule to generate a double gradient for fluid movement in the microcirculation. Therefore, we hypothesized that PEG-20k in shock acts via biophysical effects on water movement in the microcirculation through both cell impermeant and oncotic properties. These properties prevent cell swelling during ischemia, reload the capillaries with isotonic fluid from the interstitial space, and decompress the microcirculation, which all leads to increased capillary perfusion and oxygen transfer in the low volume state. Figure 1 shows the hypothesized biophysical mechanisms of PEG-20k based LVR solutions in low flow and shock states. This study presents the results of experiments that support this hypothesis.

Methods

All animal work was conducted under a protocol approved by the VCU Institutional Animal Care and Use Committee, which is governed by the rules and regulations set forth in the NIH guide and the USDA.

Rodent Shock Model

A low volume resuscitation (LVR) model was used in adult rats to test the impermeant-based LVR solutions used for pre-hospital resuscitation during severe hemorrhagic shock (1, 15). Adult male Sprague Dawley rats were anesthetized and maintained in a light surgical plane of anesthesia with isoflurane during the study. Isoflurane was delivered through a nose cone with a fraction of inspired oxygen of 100%. The animals were allowed spontaneous respirations to control their own ventilation and carbon dioxide levels. Polyethylene catheters were placed in both femoral arteries for blood pressure monitoring and blood sampling, and a third catheter was placed in a femoral vein for fluid administration. Heparin (500 U/kg) was given intravenously (IV) to maintain catheter patency. A one centimeter midline incision was created to induce some soft tissue injury and for placement of an intra-abdominal temperature probe. Animals were kept at 38°C using a heating pad and an incandescent light source. Arterial blood pressure, heart rate, and temperature were continuously recorded using PowerLab (ADInstruments, Boston, MA).

After a 15 minute stabilization period, arterial blood was removed at 1 ml/min into a syringe to maintain a mean arterial pressure (MAP) of 30-35 mmHg. More blood was withdrawn as the animal compensated, but a maximum hemorrhage limit of 60% of blood volume was set. Blood volume (ml) was estimated as: (weight (g) x 0.06) + 0.77 as previously described (16). A MAP of 30-35 mmHg was maintained until the plasma lactate reached a value

between 9-10 mM, as measured every 15 minutes with a hand held lactate analyzer (Lactate Plus, Nova Biomedical, Waltham, MA) and every hour with the ABL-800 blood gas analyzer (Radiometer, Copenhagen, Denmark). Once the target lactate was reached, a low volume resuscitation equal to 10% of the estimated blood volume was given IV over five minutes using a syringe infusion pump. Thirty minutes after LVR, serial lactate measurements were taken until the lactate again climbed back to the 9-10 mM target because the low volume infusions temporarily lower or stall the accumulation of plasma lactate. The main outcome measured was LVR time, which was defined as the length of time from the start of LVR administration to the time when lactate climbed back to 9-10 mM since lactate often falls temporarily after administration of volume due to increased oxygen delivery and dilution. The LVR time is a surrogate for tolerance to the low volume or shock state. It is the length of time that a patient can safely remain in the low volume state until definitive care and resuscitation are needed, clinically, the "Golden Hour". At the end of the experiment, the animals were euthanized by Euthasol injection or by exsanguination under anesthesia. Figure 2 depicts the experimental protocol.

Fluids used for low volume resuscitation were: 1) Normal saline, 2) Cell impermeants alone: 15% gluconate or equal volumes of 15% gluconate, 35% raffinose and 25% trehalose in saline, 3) 10% albumin in saline, 4) 10% PEG-20k in saline, and 5) 10% gluconate + 10% albumin in saline. Other outcomes recorded included the lactate at the end of the LVR time, which in most cases was 9-10 mM by definition. Mean arterial pressure (MAP) was also recorded throughout the experiment.

Regional Blood Flow

In another series of studies (n = 5), local capillary blood flow was studied using the

colored microsphere technique (1, 17). Animals were prepared as previously described but a catheter was also placed into the aortic root, using real time pressure and pressure waveforms as indicators of catheter tip location by identifying the aortic valve. During the stabilization/baseline period, 300 μL colored microspheres (Triton Technologies, San Diego, CA) were rapidly injected into the aortic root as a calibrated arterial reference blood sample was simultaneously removed from the femoral artery catheter with a withdrawal pump at a constant rate of 0.25 ml/min. A different color microsphere was injected 30 minutes after LVR. After the study, tissue samples were removed from major organs, and microspheres were recovered from the tissue samples and reference arterial blood samples by alkaline digestion and repeated centrifugations. Dye coating the purified colored microspheres was extracted with acidified 2-ethoxyethyl acetate and quantitated using a UV-VIS spectrophotometer (Shimadzu). Individual colors were resolved using a matrix inversion algorithm from the composite spectra. Blood flow was calculated by the tissue dye content using the reference blood draw as a blood flow standard. Correction for microsphere loss occurred using the recovery of blue microspheres that were added to the tissues as an internal standard prior to digestion (10,000 spheres added per sample). All flows were normalized to 100 g tissue weight and expressed as the change from baseline values before shock and low volume resuscitation.

Blood Volume Determinations:

Total blood volume of rats after shock and after various times following LVR were calculated using the indicator dilution technique (18, 19). An I.V. bolus of FITC-Albumin (Sigma-Aldrich, St. Louis, MO) of known volume and activity was administered and a reference I.V. sample was taken 15 minutes later for estimation of the volume dilution effect. Plasma volume was calculated by the degree of FITC dilution using a standard dilution curve with

saline. Plasma volume was divided by 1-Hct to determine the circulating blood volume. Blood volume was also assessed by the same indicator dilution principle using hematocrit during the LVR period. The assumptions of this method were: 1.) The red blood cells stay in the vascular compartment during LVR; 2.) The volume of the packed cell component remains constant during LVR (because no further bleeding is allowed), and; 3.) Changes in Hct during LVR are inversely proportional to changes in the plasma volume component of the intravascular space. Baseline blood volumes before shock were estimated using a formula as previously described (1).

Statistical Analysis

Data are expressed as mean ± standard deviation. Each group consisted of 5-9 rats, which was derived from power analysis and the known variance of the data from similar studies. Data were analyzed by one-way or two-way analysis of variance (ANOVA) and Bonferroni's multiple comparison correction using the InStat program (GraphPad Software, Inc., La Jolla, CA). A *p*-value < 0.05 was considered statistically significant.

Results

The effects of a variety of chosen LVR solutions on LVR time are shown in Figure 3. Normal saline was the control LVR fluid, which produced a LVR time of 34 ± 8 minutes. The LVR time significantly increased to 114 ± 10 minutes and 92 ± 20 minutes in the impermeant and albumin groups, respectively, compared to saline. The LVR time for the PEG-20k group was 240 ± 0 minutes ($p < 0.05$, relative to saline, impermeant, and albumin groups). This LVR time

was cut off for technical reasons and would have been higher because the lactate at 240 minutes was only 1.2 mM, which is well below the target cutoff of 9-10 mM. There was no significant difference in LVR times between PEG-20k and the albumin + gluconate treated groups (240 min and 225 min, respectively). However, the true comparison between these two groups is unknown because the full LVR time in the PEG-20k group was not realized because the lactate target was not reached. This was technically the result of time dependent anesthesia problems in the animals after 4 hours. Therefore, the reported magnitude of the PEG-20k effect during shock in Figure 3 is underestimated when measured by the LVR time.

Similar to the LVR times, the mean arterial blood pressure in the impermeant, PEG-20k, and albumin groups were much higher throughout the LVR period, compared to the saline LVR control (Figure 4). Generally, the mean arterial blood pressures during LVR correlated with the LVR times such that the groups with the longest LVR time (PEG-20k) also had the highest MAP and vice-versa (Figure 4).

Reductions in capillary blood flow after shock and LVR, as measured by the colored microsphere technique, were significantly less in all organs and tissues (except the ileum) during the LVR period in PEG-20k resuscitated animals, relative to the saline resuscitated control animals (Figure 5). All flow values (except in the left ventricle) in the saline group after LVR were statistically lower than their paired baseline values and all flow values in the PEG-20k group after LVR were statistically unchanged from their paired baseline values. In other words, shock and LVR with saline caused significant reductions in local tissue blood flow, which was prevented when PEG-20k was used as the LVR solution.

Blood volume measurements made after hemorrhage and at 15, 30, and 60 minutes after LVR administration in saline and PEG-20k groups are shown in figure 6. Blood volume was estimated prior to shock. Shock significantly reduced blood volume in both groups, relative to baseline. Resuscitation with PEG-20k significantly increased blood volume at all times after LVR compared to the saline control LVR solution using either indicator dilution technique. However, the steady increase in blood volume seen during LVR in the saline group using the FITC-albumin method disappeared using the Hct method. Specifically, resuscitation with low volumes of 10% PEG-20k, but not saline, caused blood volume to significantly increase above values observed after hemorrhage.

Discussion

Our previous studies have demonstrated efficacy of a novel platform of low volume resuscitation crystalloid solutions in extending the tolerance to the low volume state or the amount of time that a severely shocked patient can safely remain in the low volume state until definitive resuscitation and medical care are delivered. These solutions contain cell impermeants and are designed specifically to reduce the amount of ischemia-induced cell swelling during shock. This effect directly protects tissues from the adverse effects of cell swelling per se and decompresses the microcirculation during shock, which re-establishes capillary flow in the periphery. Low volume resuscitation solutions that used the specific polymer PEG-20k produced striking biological effects that were multiple fold greater than the salutary effects seen with previous classes of smaller cell impermeants. The objective of this study was to explore the

mechanisms of action of PEG-20k that account for these strong biological and pre-clinical effects.

Simple cell impermeant molecules like gluconate, trehalose, and raffinose have been used in organ preservation solutions to prevent tissues from swelling in cold ischemic environments. Ideal cell impermeants are molecules that have a unique size enabling them to freely escape the capillary space but not cross the cell membrane because they are too big or charged. Thus, they accumulate outside of the cell and osmotically hold water from entering the cell, which is its normal propensity during shock and ischemia when energy dependent volume control mechanisms fail (Na^+/K^+ ATPase). These simple impermeants prevented cell and tissue swelling in rodent models of hemorrhagic shock when introduced into low volume resuscitation solutions. Their low toxicity and chemical inertness allows them to be successfully used in high concentrations capable of exerting these necessary biophysical effects on water shifts during shock. Cell impermeants quadrupled the low volume resuscitation time compared to saline. In an attempt to optimize this effect, we added colloidal molecules to the impermeants to create a second osmotic gradient in the microcirculation, which was designed to pull water into the capillary space. The first studies used PEG-20k as a colloid together with the simple impermeant gluconate. This increased the LVR time 7-fold in the rodent model, which suggested that the double gradient approach may have worked. However, subtraction experiments using only PEG-20k without the gluconate (impermeant) produced the same effect as the two together. It was then hypothesized that the larger PEG-20k molecule may be acting as both a cell impermeant and a colloidal molecule.

Further studies indeed determined that this was true since the osmotic reflection coefficient for PEG-20k was determined to be 0.5 in the rat microcirculation (thoracic and

mesenteric beds) under non-shock conditions (15). This means that roughly 1/3 of the PEG-20k escapes the capillary space to load into the interstitium where it acts like a simple cell impermeant (gluconate) and 2/3 of the molecules in the circulation remains behind in the capillary space where it acts as a colloidal agent to produce the second osmotic gradient. While this double gradient effect of PEG-20k is completely and unambiguously supported by the biophysical osmotic reflection coefficient data, the translation of these properties into the strong biological effects seen with PEG-20k remain less certain. To make this biological link and support the hypothesis that the second osmotic gradient indeed contributes to the very long LVR time of PEG-20k during shock, we attempted to recapitulate the biological effect of PEG-20k with two distinct molecules used together: a small ideal cell impermeant (gluconate) that produces an osmotic gradient between the intracellular and the extracellular space and a classic colloidal agent (albumin) that produces a second osmotic gradient between the interstitial space and the capillary (intravascular) space.

Indeed, the use of these two distinct molecules (albumin and gluconate) produced a biological effect during low volume resuscitation that was similar as PEG-20k when used alone. This supports the hypothesis that the biological effect seen with PEG-20k may be due, in part, to its unique biophysical attributes that allow it to behave as both an ideal impermeant and a colloid in the microcirculation during shock states. However, since we do not know the exact LVR time with PEG-20k alone because the period was terminated early, biological differences between this group and the albumin + gluconate group probably exist, which suggests that not all of the PEG-20k effect may be attributable to osmotic gradients. While other biological effects of PEG-20k are likely in these settings, they currently remain unknown. Protection and hydration of the

shock-eroded glycocalyx by PEG-20k polymers remains a strong possibility that is under investigation.

Rats receiving saline had a transient increase in MAP during LVR infusion, but the pressure started to drop as soon as the infusion stopped. This is because saline physiologically distributes unequally between the vascular and interstitial compartments. About 20% of the administered saline volume will remain in the intravascular space and 80% goes elsewhere (20, 21). Since only the 20% remaining in the capillaries supports arterial pressure, it is not surprising that saline given in low volumes during shock produce poor or absent effects on the arterial pressure. When PEG-20k based LVR solutions are administered, it may prevent saline from loading into the interstitium while simultaneously returning the fluid that leaked into the interstitium back into the capillary spaces during the shock period (22-24). These passive volume shifts reduce local tissue swelling, decompress the microcirculation, reduce resistance to flow, and reload the capillaries. All of these changes drive local tissue perfusion and provide cardiac preload. This was demonstrated directly by the increase in MAP and indirectly by the rapid clearance of lactate in LVR and by direct measurements of increased capillary blood flow with PEG-20k compared to saline. This is further supported by the significant effects of PEG-20k solutions on expansion of the plasma and blood volumes after their administration during the LVR period. PEG-20k maintenance of perfusion pressure and lactate clearance in the low volume state can extend up to eight hours (unpublished data), which was the longest period so far examined.

Plasma volume expansion occurs with PEG-20k LVR solutions as indicated by indicator dilution studies. Curiously, the FITC-albumin method suggests volume expansion throughout the one-hour LVR period after saline administration. We suspected this to be an

artifact of the calculations when a shock-induced capillary leak occurs that allows the albumin tracer to escape into the interstitial space. This would appear as a dilution from intravascular volume expansion when in fact the escaped tracer is only labeling a nonvascular space. To test this, we calculated intravascular volume during the LVR period in both groups using the hematocrit as the fixed volume intravascular indicator because some important assumptions in the model were met. The volume calculations based on hematocrit dilution seem to unmask a small artifact in the FITC-albumin dilution calculations, which most likely indicates capillary leak associated with the low volume and ischemic state.

The maintenance of near normal MAP in this study are salutary because this is a controlled model of hemorrhagic shock. In most real trauma settings, uncontrolled bleeding occurs in abdominal and thoracic spaces that are exacerbated by high or near normal pressure. This could be a problem in uncontrolled hemorrhagic shock because the higher MAP associated with PEG-20k LVR would cause more direct pressure related bleeding and indirectly by a “pop the clot” mechanism. In preliminary and yet unreported studies, the pressure response in a porcine shock model to PEG-20k based LVR solutions was less robust (around 60 mm Hg), relative to rats (100 mmHg). This may be preferential in clinical shock if humans respond to I.V. PEG-20k solutions more like pigs and less like rodents. Such a permissive hypotension may be the sweet spot between adequate tissue oxygen transfer and exacerbation of uncontrolled bleeding in the field.

Hemorrhagic shock decreases oxygen delivery, which results in the accumulation of oxygen debt during the low volume state. Using lactate clearance directly and the LVR time indirectly, which relies on lactate levels, we were able to clearly demonstrate that PEG-20k based LVR solutions both stop accumulation of oxygen debt and rapidly repay the debt even during the low volume state. This is supported by the rapid drop in lactate levels after PEG-20k

based LVR and the extremely long LVR times, relative to the values seen with conventional saline LVR. While about 50% of this drop in lactate can be attributable to dilution from an expanding intravascular volume, much of the remaining lactate clearance may be due to increased efficiency of microvascular capillary oxygen transfer and/or an overall increase in oxygen delivery, which drives the conversion of lactate back to pyruvate for subsequent aerobic ATP synthesis. In fact, preliminary studies in a large animal porcine model of shock and low volume resuscitation indicate that PEG-20k based LVR under similar low volume conditions leads to a hyperdynamic cardiovascular response characterized by cardiac output increasing 50% higher than pre-shock baseline values over much of the low volume state (unpublished observations). These combined factors likely account for the apparent rapid oxygen debt repayment and the 100% overnight survival (1) seen in PEG-20k treated rodents. In patients with long pre-hospital transport times, this can limit further ischemic and reperfusion injury and possibly begin debt repayment during the transport period.

Other possible mechanisms of action of PEG-20k polymers on low flow shock outcomes include drag reduction in the circulation. Although soluble drag reducing polymers have been shown to have positive effects in shock, a similar mechanism with PEG-20k is doubtful since the molecular weight cut-offs for drag reduction effects seems to be 1,000 kDa, which is far below the 20 kDa of the polymers used in this study (25).

A likely mechanism of action of PEG polymers in LVR not addressed by this study include reconstruction of the endothelial glycocalyx by PEG-20k. Shock and crystalloid resuscitation are known to erode the glycocalyx, thus promoting resuscitation injury by promoting cellular inflammation (26, 27). Polyethylene glycol polymers are known to bind to the cell membrane with their accompanying water layers (28) that could effectively rebuild the

glycocalyx during the low volume resuscitation and reperfusion period (29). While this likely happens, it seems that such effects would be expressed after longer periods of resuscitation since cellular inflammation may require hours rather than minutes, which is the time period where rapid capillary blood flow and lactate clearance were observed with PEG-20k in this study. It is reasonable to suggest that PEG-20k LVR may reload peripheral capillaries by early osmotic water transfer while having later effects on glycocalyx-mediated cellular inflammation.

In conclusion, 10% PEG-20k is a novel low volume resuscitation fluid with encouraging potential for pre-hospital use in hemorrhagic shock. By improving local oxygen delivery and capillary perfusion, these solutions increases tolerance to the shock state during prolonged pre-hospital and transport periods. This study tests a likely biophysical mechanism for its efficacy, namely, the osmotic cell-to-capillary transfer of accumulated water that drives efficient local perfusion under low volume conditions.

Figure Legends

Figure 1. Hypothetical mechanism of action for PEG-20k in low volume resuscitation for shock. Cell impermeants can escape the capillary but are too large or charged to enter cells, and thus create an osmotic gradient to prevent cell swelling during shock. We hypothesized that PEG-20k acts via biophysical effects on water movement through both cell impermeant and oncotic properties. To test our hypothesis, we tried to recapitulate the PEG-20k effect by combining a cell impermeant (gluconate) with a colloid (albumin). The movement of capillary water with oncotic agents increases capillary pressures that promote capillary flow even under low-volume states. The sum effect is to promote effective and efficient capillary transport and oxygen delivery in the low-volume state. I.S., Interstitial space.

Figure 2. Timeline diagram of the hemorrhagic shock and resuscitation protocol. Rats were hemorrhaged to a mean arterial pressure of 30-35 mmHg. Once lactate reached 9-10 mM, low volume resuscitation (LVR) was administered. The primary outcome was LVR time, which is the length of time from start of LVR to the time at which lactate climbed back to 9-10 mM.

Figure 3. The effect of different LVR solutions on LVR time. Data are presented as mean (SD). Numbers below bars indicate mean lactate at the end of LVR time, which by definition should be 9-10 mM. Numbers above bars indicate sample size. All the treatment groups had significantly higher LVR times than the saline (control) group. There was no significant difference between PEG-20k and the albumin + impermeant group.

Figure 4. Mean arterial pressures after LVR administration, measured at 15 minutes, 30 minutes, throughout the LVR period, and at end of LVR time. * $p < 0.05$ relative to the other corresponding groups, # $p < 0.05$ relative to all other corresponding values.

Figure 5. Capillary blood flow measured 30 minutes after LVR. Data are presented as mean (SD) as % change from baseline. Each rat served as its own baseline. * $p < 0.05$ compared to saline group. For the saline group, all flows except left ventricle were statistically less than paired baseline flows. For PEG-20k, all flows were not different from baseline. n = 5.

Figure 6. Circulating blood volume measured in rats by the indicator dilution technique using a FITC-labelled albumin probe (A) or hematocrit (B) to estimate the size of the intravascular fluid compartment during low volume resuscitation with saline or saline containing 10% PEG-20k. Blood volume values were estimated in the rats before shock (Baseline, BL), and measured with indicator dilution after the hemorrhagic shock (HS) period, and after the low volume resuscitation (LVR) period. Values are mean ± SD for 4 rats in each group. * P<0.05 relative to the corresponding value in the saline group. Both LVR solutions were given at a volume of 10% of the estimated baseline blood volume. Saline was 0.9% NaCl solution and PEG-20k was a 10% weight to volume solution of polyethylene glycol (mean mw = 20,000 Da) dissolved in saline.

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Figure 1

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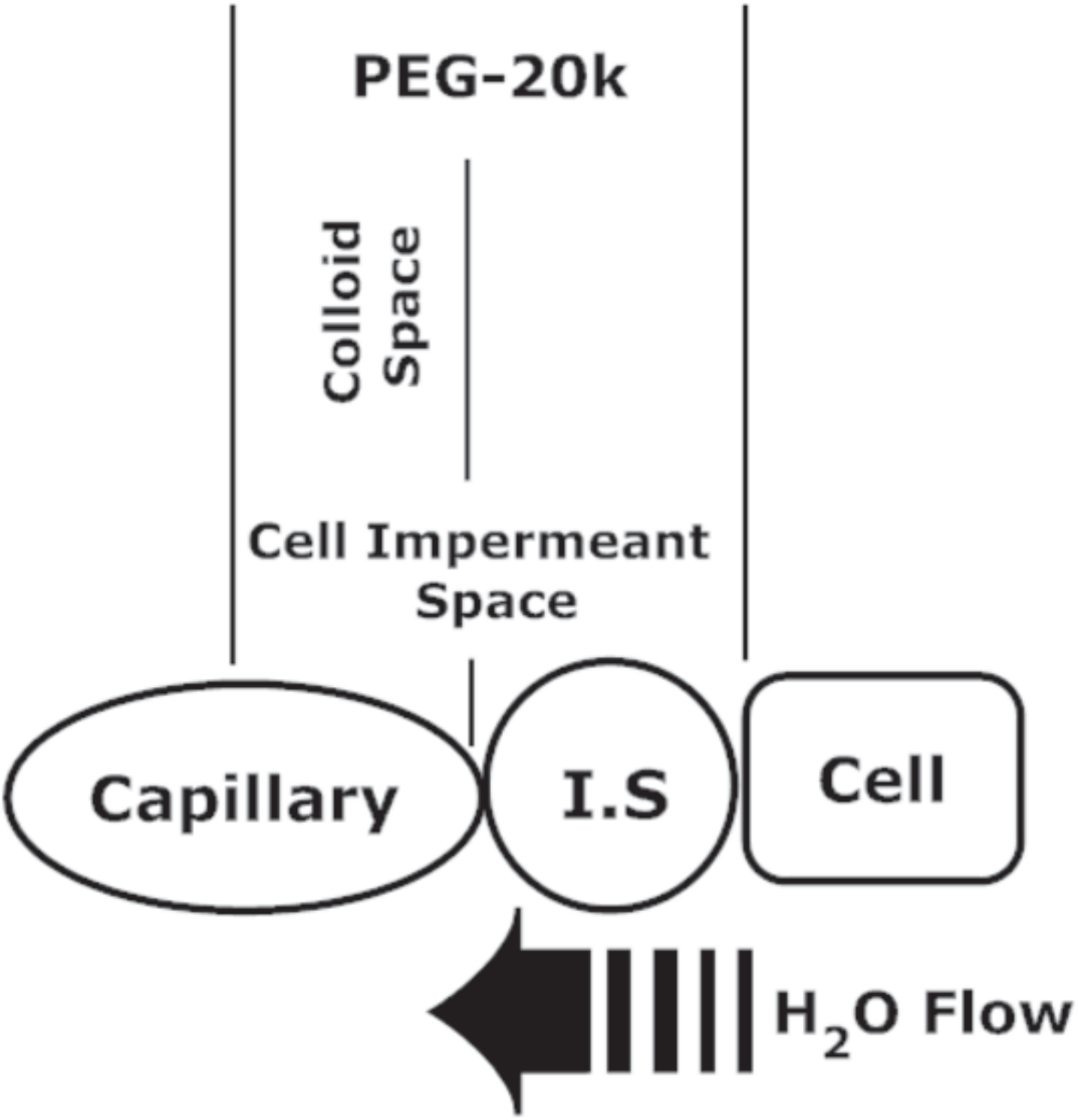

Figure 2

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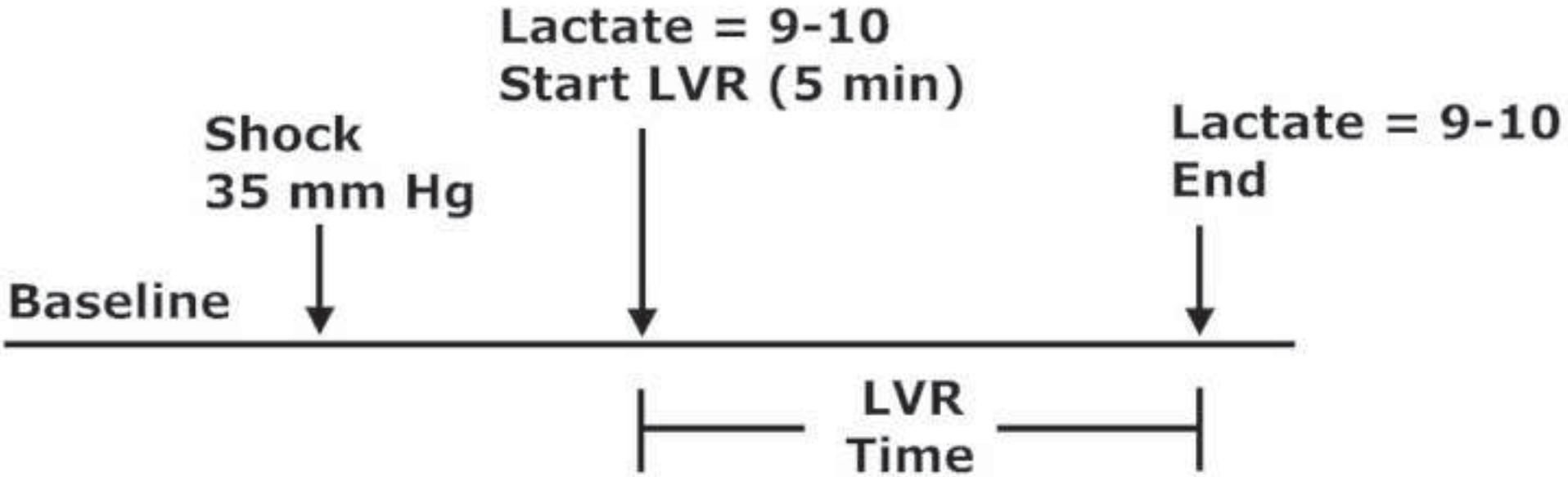
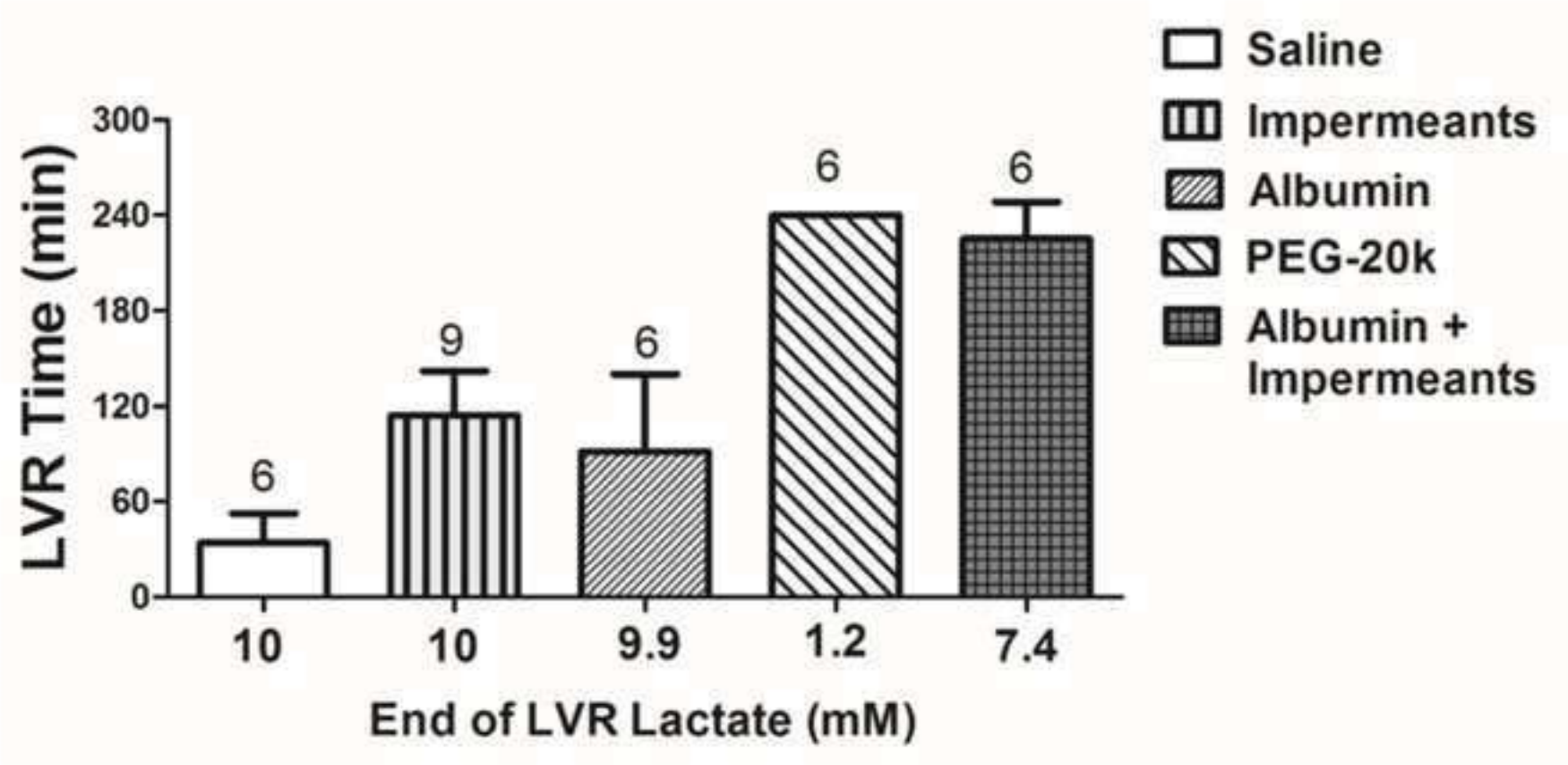


Figure 3



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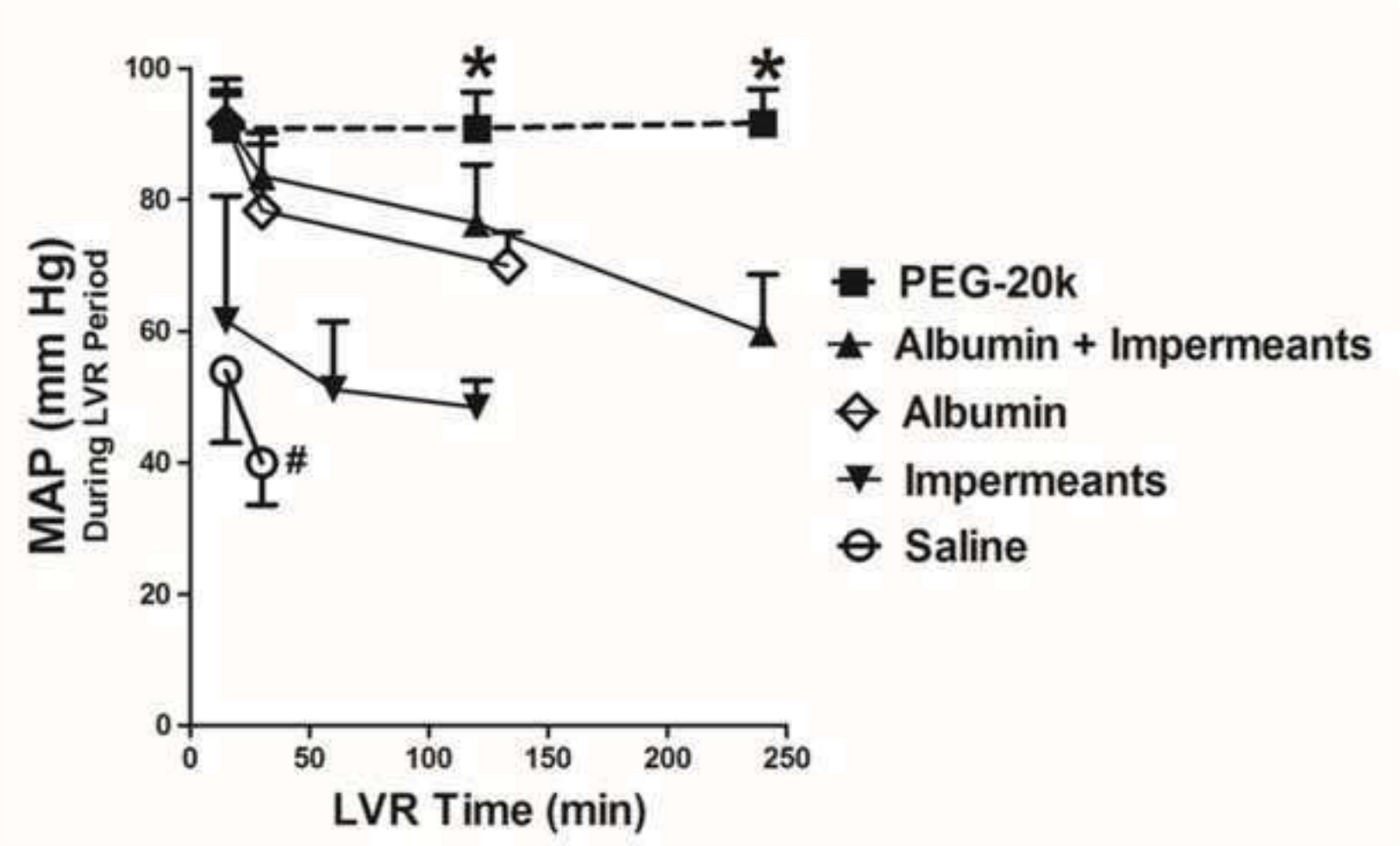

Figure 5

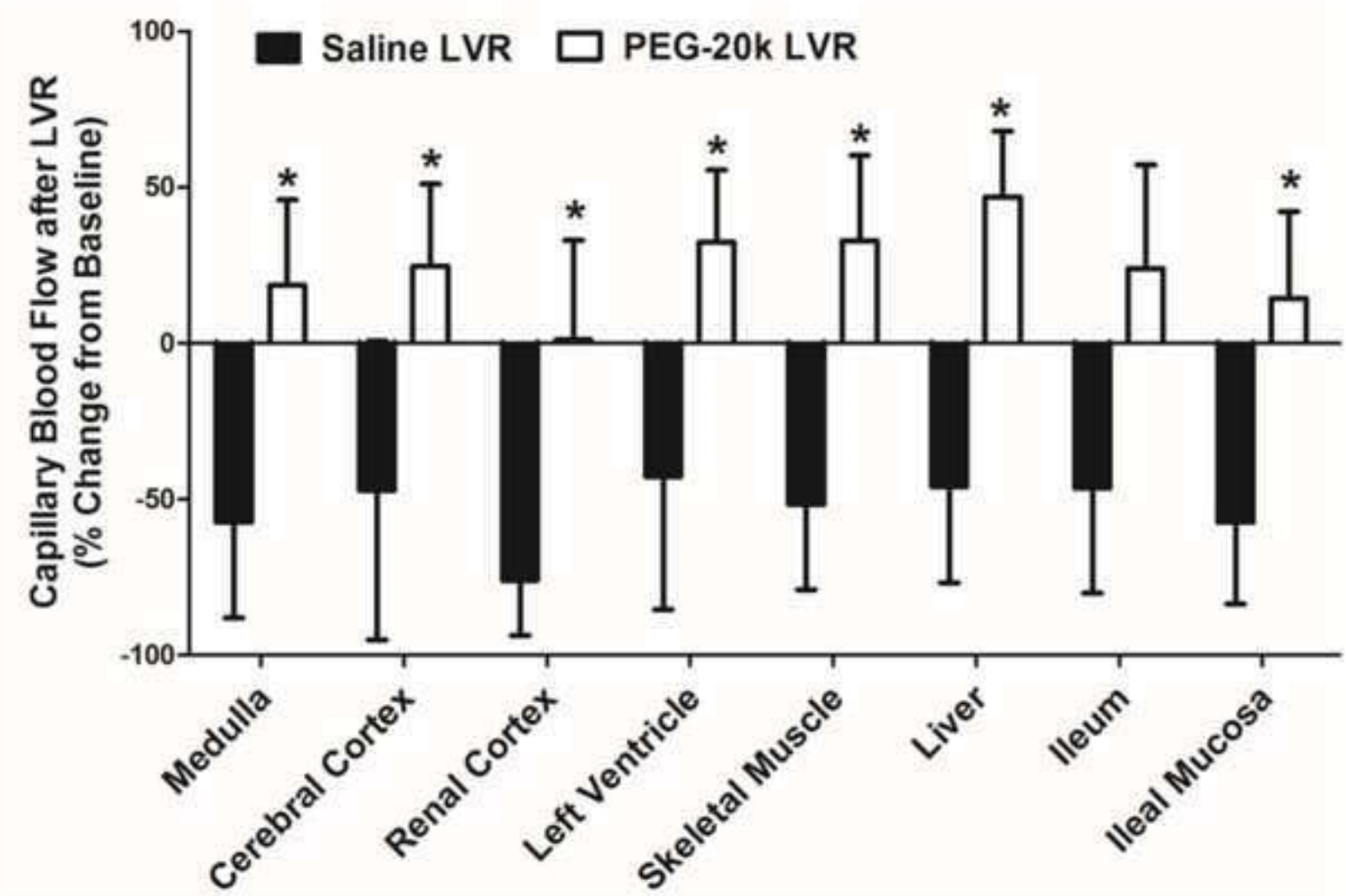

Figure 6

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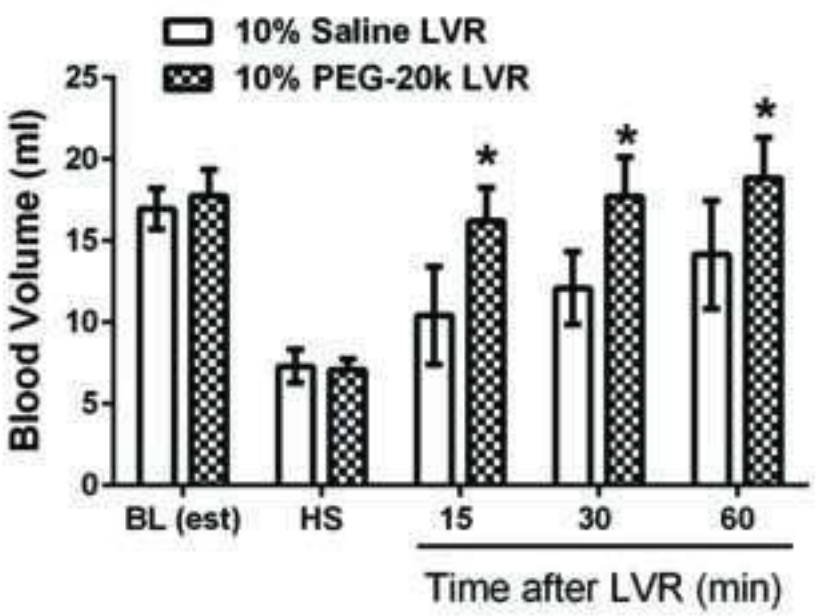
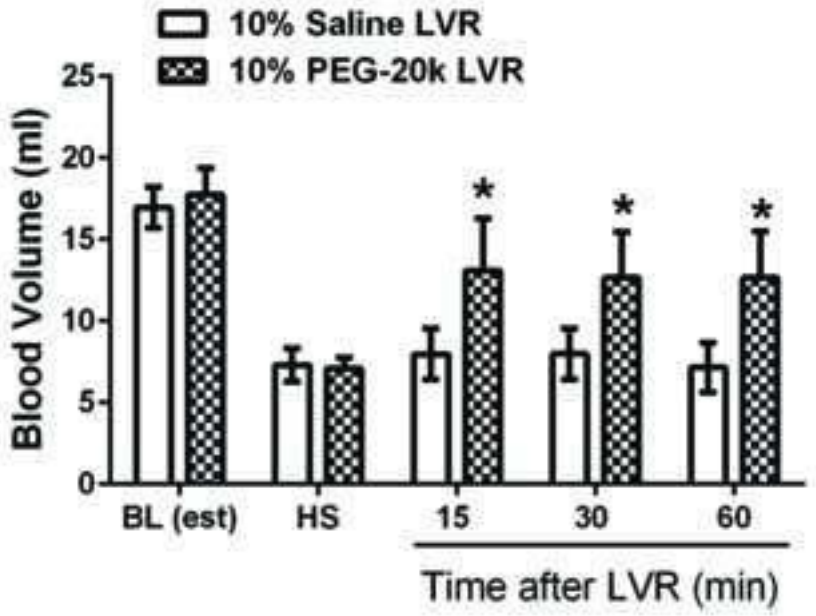
A. FITC-Albumin Method



B. RBC Method



Abstract

Introduction: Polyethylene glycol-20k (PEG-20k) is highly effective for low volume resuscitation (LVR) by increasing tolerance to the low volume state. In our rodent shock model, PEG-20k increased survival and expanded the "golden hour" 8-fold compared to saline. The molecular mechanism is largely attributed to normalizations in cell and tissue fluid shifts after low flow ischemia resulting in efficient microvascular exchange. The objective of this study was to evaluate PEG-20k as a low volume resuscitation solution for hemorrhagic shock in a pre-clinical model.

Methods: Anesthetized male Yorkshire pigs (30-40 kg) were hemorrhaged to a MAP of 35-40 mmHg. Once lactate reached 7 mM/L, either saline (n = 5) or 10% PEG-20k (n = 5) was rapidly infused at 10% calculated blood volume. The primary outcome was LVR time, defined by the time from LVR administration to the time when lactate again reached 7 mM/L. Other outcomes measured included: MAP, heart rate (HR), cardiac output (CO), mixed venous oxygen saturation (SvO2), splanchnic blood flow, and hemoglobin.

Results: Relative to saline, PEG-20k given after controlled hemorrhage increased LVR time by 15-fold, a conservative estimate given that the lactate never rose after LVR in the PEG-20k group. Survival was 80% for PEG-20k LVR compared to 0% for the saline controls ($P<0.05$). PEG-20k also significantly decreased HR after hemorrhage and increased CO, MAP, splanchnic flow, and SvO2. Falling hemoglobin concentrations suggested sizable hemodilution from fluid shifts into the intravascular compartment.

Conclusions: In a pre-clinical model of controlled hemorrhagic shock, PEG-20k-based LVR solution increased tolerance to the shock state 15-fold compared to saline. PEG-20k is a superior crystalloid for low volume resuscitation that may increase safe transport times in the prehospital setting and find use in hospital emergency departments and operating rooms for patients awaiting volume replacement or normalization of cell, tissue, and compartment fluid volumes.

Low Volume Resuscitation Using Polyethylene Glycol-20k in a Pre-Clinical Porcine Model of Hemorrhagic Shock

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Key Words: Osmotic changes, microcirculation, reperfusion, polymers, crystalloid

Author Contribution: Each author contributes to the background and design of the studies, to some aspect of the execution of the study, and to data analysis and manuscript preparation.

Introduction

Hemorrhage causing severe tissue hypoxia is a leading cause of death in military and civilian populations (1-3). Yet care for wounded military combatants is quite different compared to civilian patients and is limited by available resources and delayed evacuation or prolonged transport to definitive care (2;4). Medical supplies carried in the field must be of low weight, chemically stable, and small volume (2). The recommendation for hemorrhage shock in *Fluid Resuscitation*, a report published by the Committee on Fluid Resuscitation for Combat Causalities and Institute of Medicine in 1999, is a 250 ml bolus of 7.5% saline. However, a subsequent randomized, double-blinded study by the Resuscitation Outcomes Consortium did not show improved survival outcomes after treatment with hypertonic saline (5;6). In fact, hypertonic saline worsened hypo-coagulation and hyper-fibrinolysis (7). There remains a need for an ideal fluid that can be given at a low volume to resuscitate patients in hemorrhagic shock awaiting definitive treatment, especially in the austere prehospital setting.

Parrish et al. have demonstrated reduced ischemia-induced cell swelling, increased tolerance to the low volume state, improved capillary blood flow, and higher survival with administration of a low volume solution containing polyethylene glycol-20k (PEG-20k) in a rodent model of severe hemorrhagic shock (8;9). The resuscitation effects were extraordinary in this model. These effects are attributed to the specific polymer radius and osmotic reflection coefficient that accelerate non-energetic fluid movement into capillaries by multiple osmotic gradients. The next step was to test PEG-20k in a pre-clinical porcine model. The objective of this study was to establish preliminary data for PEG-20k use as a low volume resuscitation agent in a pre-clinical porcine model of hemorrhagic shock. We hypothesized that pigs in hemorrhagic shock receiving PEG-20k would have increased tolerance to the shock state, as measured by the low volume resuscitation (LVR) time, compared to pigs receiving saline. We also hypothesized that PEG-20k treated pigs would have improved cardiovascular outcomes relative to saline-treated pigs.

Methods

All animal work was conducted under a protocol approved by the VCU Institutional Animal Care and Use Committee, which is governed by the rules and regulations set forth in the NIH guide and the USDA. The procedures were generally as described previously, except a lactate controlled endpoint was used (10).

Male Yorkshire pigs (30-40 kg, Archer Farms) were fasted the night before the experiment, but access to water was allowed. Anesthesia was induced with an intramuscular injection of ketamine and Xylazine and intravenous injection of propofol. After endotracheal intubation, isoflurane at 1-2% was used to maintain anesthesia with a fraction of inspired oxygen of 30% (Narkomed 2 Ventilator, Drager, Lubeck, Germany). If needed, a circulating water warming pad was used to maintain temperature of 38°C.

Once appropriate anesthesia was achieved, vessel cannulations were performed using cut-down method. The right femoral artery was cannulated with a (PE 280) cannula and was connected to a

hemodynamic monitoring system (PowerLab, ADInstruments, Boston, MA). The right carotid artery was cannulated for blood withdrawal. The right external jugular vein was cannulated with a 7 Fr. pulmonary artery catheter (Swan-Ganz Continuous Cardiac Output Thermodilution Catheter, Edwards Lifesciences, Irvine, CA) with the tip in a wedge position when the balloon was inflated. The PowerLab data acquisition system was used to monitor pressure waveforms during pulmonary artery catheter placement. The left external jugular vein was cannulated for fluid administration. A midline abdominal incision from xiphoid to pubis was then made to cause a measured surgical trauma injury and to gain access to a ~2 mm branch of the superior mesenteric artery. After careful dissection, a flow probe was placed around the artery and connected to transit-time blood flowmeter (Transonics model T403, Ithaca, NY) that was monitored by the PowerLab. For three animals, pulmonary artery catheterization and/or mesenteric artery dissection were not performed due to technical complications. Arterial blood pressure, heart rate, mesenteric artery flow, and temperature were continuously monitored by PowerLab, and pulmonary artery catheter variables, including mixed venous oxygen saturation (SvO_2) and cardiac output, were continuously measured by a cardiac output computer (Edwards Lifesciences Vigilance Irvine, CA).

In most animals, a pre-hemorrhage saline bolus was administered intravenously until the capillary wedge pressure reached 5-8 mmHg. This was done to normalize baseline volumes before hemorrhage. An arterial blood gas (ABG) sample was obtained, and adjustments were made to the ventilator to maintain pCO_2 between 38-42 mmHg. Additional ABG samples were obtained if needed to assess interventions.

After a 15 minute stabilization period, arterial blood was removed at an initial rate of 4 ml/kg using a Masterflex® peristaltic roller pump (Cole-Parmer, Chicago, IL) until the mean artery pressure (MAP) reached 35-40 mmHg. More blood was withdrawn at a subsequent rate of 2 ml/kg as the animal compensated. A MAP of 35-40 mmHg was maintained until plasma lactate reached a value between 7-8 mM/L, as measured every 15 minutes with a hand held lactate analyzer (Lactate Plus, Nova Biomedical, Waltham, MA) and with the ABL-800 blood gas analyzer (Radiometer USA, Cleveland, OH). Once the target lactate was reached, a low volume resuscitation (LVR) solution equal to 10% of calculated blood volume of either saline (n = 5) or 10% w/v PEG-20k (n = 5) was given IV over 5 minutes using a Masterflex® roller pump. Circulating blood volume was estimated as 70 ml/kg body weight.

Fifteen minutes after LVR, serial lactate measurements were taken until the lactate again climbed to the 7-8 mM/L target. The main outcome was LVR time, which was defined as the length of time from start of LVR administration to the time at which lactate again reached 7-8 mM/L. If lactate did not decrease after LVR and only increased in subsequent measurements, LVR time was arbitrarily set at 15 minutes, which was the minimum time sampling interval. If lactate decreased and did not reach the target of 7-8 mM/L by 240 minutes, the maximum LVR time was arbitrarily set at 240 minutes. The LVR time is a surrogate for tolerance to the low volume or shock state (8;9). Clinically, it would be similar to the length of time that a patient can safely remain in the low volume state until definitive care and full resuscitation are needed, i.e. the

"Golden Hour." At the end of the experiment, animals were euthanized by administration of I.V. Euthasol.

Data are expressed as mean ± standard deviation. Data were analyzed by two-way ANOVA and Bonferroni's multiple comparison correction and the Fisher's Exact test using the InStat program (GraphPad Software, Inc., La Jolla, CA). A *p*-value < 0.05 was considered statistically significant.

Results

The consistency of the two groups of animals used in the study is shown, in part, by the similarities in the times required to complete milestones during the protocol. Figure 1 shows protocol times for the control and the experimental LVR groups for surgery, fluid loading, establishment of baselines, total preparation time, time to achieve equal degrees of oxygen debt during hemorrhage, and the low volume resuscitation times. All times were similar between the two groups except the LVR time where the PEG treated pigs had a significantly higher time, compared to the controls (* P<0.05). Other demographic values and characteristics were identical between the two groups of pigs.

Survival in the saline group 0% (0/5) was significantly lower than the survival in the PEG-20k treated group 80% (4/5), as determined by Fisher's Exact test (P = 0.047).

The effects of hemorrhage and low volume resuscitation on plasma lactate concentrations in the two pig groups are shown in Figure 2A. Lactate in both groups rose steeply at the same rate during hemorrhagic shock to reach the predetermined target and then continued to climb after low volume resuscitation with saline. However, LVR using PEG-20k caused the plasma lactate concentrations to steadily decline over the next 4 hours to end at values that were the same as the baseline before hemorrhage. The low volume resuscitation (LVR) time, which is derived from the plasma lactate values during the LVR period, is shown in Figure 2B. By definition, the LVR time is the time required for the plasma lactate to reach 8 mM after giving the LVR solution. In saline pigs, the plasma lactate never declined after LVR so we assigned an LVR value of 15 minutes, which is our shortest monitoring interval during this phase of the study. By contrast, LVR time in the PEG-20k group was 240 minutes or 16 fold higher than the control. Since the lactate in that group fell and never rose, the real LVR time was much greater than 240 min, which was arbitrarily cut off for technical reasons. In fact, the end lactate after LVR was only 1.3 mM, far less than the end point of 8 mM. The end lactate value in the saline group was 11 mM, far higher than the 8 mM target.

In addition to extending the tolerance to the low volume state (LVR time), PEG-20k produced profound effects on cardiovascular performance in pigs after low volume fluid resuscitation. Figure 3 shows that mean arterial blood pressure (MAP) and cardiac output significantly increased after PEG-20k LVR, relative to the saline control group. Heart rate (HR) significantly declined in the PEG-20k group, relative to control. Absolute MAP values settled in at about 60 mmHg in the PEG-20k group and below 30 mmHg in the saline group, which was fatal.

Mixed venous blood oxygen saturation (SvO_2) reported from the optical pulmonary artery catheter (Figure 4) fell steadily during hemorrhage but significantly increased back to baseline with PEG-20k LVR, compared to the saline LVR pigs where SvO2 continued to drop until death.

Blood flow through a branch of the superior mesenteric artery (SMA) was monitored continuously throughout these studies and is shown in Figure 5 for both saline and PEG-20k resuscitated pig groups. The mesenteric blood flow, expressed as a percentage of the pre-hemorrhage baseline, fell continuously in both groups during the hemorrhage period to about 50% of the original baseline values. After saline resuscitation, the flow remained unchanged after LVR. In the PEG-20k resuscitated group, however, mesenteric blood flow rose significantly to over 2 times the baseline blood flow after LVR. This hyperemia persisted throughout the 240 minute LVR period in the PEG-20k group.

Arterial blood gas (ABG) values are shown in Figure 6. Bicarbonate (HCO_3^-) in the arterial blood (panel A) fell during hemorrhage in both groups of pigs. Bicarbonate quickly rose to baseline values after LVR with PEG-20k compared to saline resuscitated pigs where the values remained low after LVR. The arterial CO_2 levels remained steady in both groups during hemorrhage (panel B). In the PEG-20k LVR group, the $PaCO_2$ significantly rose after resuscitation above baseline relative to the values in the saline LVR group where the carbon dioxide content continued to fall. Arterial blood pH fell in both groups during hemorrhage and normalized after LVR only in the PEG-20k group (data not shown).

Discussion

The objective of this study was to establish preliminary data for the use of PEG-20k in low volume resuscitation solutions in a pre-clinical porcine model of hemorrhagic shock. The data support our hypothesis that PEG-20k based low volume resuscitation solutions improve tolerance to the shock state as demonstrated by an increased LVR time, the surrogate for tolerance in our studies. The data also support our secondary hypothesis that cardiovascular outcomes are improved in the PEG-20k treated pigs, as compared to saline. The data are similar to the results found in the rodent hemorrhagic shock model that were previously reported by our group.

The rodent model of severe hemorrhagic shock was translated to a porcine model for pre-clinical testing of novel PEG-20k based resuscitation solutions (8;9). There are some differences between the two models. The target MAP during hemorrhagic shock was 5 mmHg higher for pigs than it was for the rats (35 Vs. 30 mmHg). This was to prevent myocardial injury in the pigs that would otherwise occur during very low MAPs (10). Plasma lactate accumulation was used as a marker for reporting oxygen debt in both models, which served as our metabolic endpoint of shock severity (11-14). The target lactates used to assess end-oxygen debt accumulation after hemorrhage was lower in pigs (7-8 mM) compared to the rat model (9-10 mM). Furthermore, the time needed to accumulate the target lactate in the pig was almost twice as long compared to the rat model (60 Vs. 115 min). These differences are attributable to differences to low volume and ischemia tolerance between the species and probably animal sizes. Some rats in the previous

studies and more pigs in the current study, failed to reach the target lactate level during hemorrhage and were not included in the study. The disassociation of oxygen debt with plasma lactate during hemorrhage in some animals underscores the shortcomings of using lactate to report oxygen debt (14). Just because lactate was not proportionally reported by some non-responding animals, does not indicate that they are not in fact accumulating oxygen debt during shock. The reasons for this limitation are unclear but it shouldn't affect the utility of the model in demonstrating differences in shock tolerance outcomes between the two treatment groups because the lactate non-responders occur randomly in both animal groups independent of the treatment and they were disqualified from the analysis because of their failure to build lactate timely. Future studies using direct oxygen debt measurements as the primary outcome are appropriate.

Hemorrhage causes oxygen debt to accumulate in patients and in animal models. This model controlled the amount of oxygen debt induced by hemorrhage by setting predetermined plasma lactate targets, after which low volume resuscitation was started. While we acknowledge problems with this technique, it attempts to ensure each resuscitated animal starts off with similar amounts of global ischemia (oxygen debt). This ensures a fair comparison of the two treatment arms if we assume that mismatches in lactate and oxygen debt occur randomly, when they do occur. The potential to re-establish oxygen exchange and transfer in the microcirculation by the type of LVR solution is indexed by the time it takes for the plasma lactate to again rise back to the target level after the start of the low volume infusion. This is defined as the LVR time and represents tolerance to the low volume state. In the saline controls, the saline LVR never reduced the rate of ascent of plasma lactate, unlike what we observed in the rat model when the saline bolus caused a temporary plateau. By contrast, pigs receiving the same volume of saline LVR containing PEG-20k continued to increase plasma lactate for the first 15 minutes after LVR but then began clearing lactate all the way to baseline. The end plasma lactate 240 minutes after LVR in the PEG treated pigs was only 1.8 mM while the lactate in the controls exceeded the target value 0-15 minutes after LVR (11 mM). Therefore, the true LVR time in the PEG group would have been much higher if the experiment were allowed to go on until the pigs began to build lactate to reach the 8 mM target. It is likely that they may never have re-accumulated enough lactate after PEG-20k LVR to reach the target value that ends the LVR period (8 mM) because their lactate values after 4 hours of hypovolemia were so low and their hemodynamic status was so stable. Therefore, the 240 min LVR time in the PEG group is arbitrary and grossly underestimates the true value. The PEG-20k crystalloid LVR alone without further resuscitation using blood products may be sufficient to induce total ischemic tolerance to the low volume state but inadequate to handle traumatic and dilutional coagulopathies. Uncontrolled shock studies in pigs will highlight the importance of combining solutions like PEG-20k that enhance tissue perfusion and oxygen transfer with blood products that control coagulation and hemostasis.

The near normalization of plasma lactate with PEG-20k based LVR solutions was attributable to both dilution and clearance. About a 50% dilution effect from the PEG-induced osmotic movement of isotonic water into the vascular space was observed. The remaining drop in plasma lactate was attributable to non-dilutional mechanisms. The hemoglobin data suggest intravascular dilution because the hemoglobin concentration after LVR with PEG-20k was about

50% of the baseline. Since no further bleeding or loss of RBCs was allowed to occur after the end of hemorrhage (because it was a controlled bleed model), the hemoglobin concentration reduction likely represents a proportional dilution effect with non-hemoglobin containing fluid from outside of the vascular compartment. The remaining 50% non-dilutional drop in plasma lactate that was observed after PEG-20k LVR likely represents a combination of clearance by renal filtration into the urine and by the metabolic oxidation of lactate to pyruvate by LDH as the peripheral microcirculation opens (8;9) and aerobic metabolism resumes.

Oxygen debt induced by severe hemorrhagic shock was not directly measured in this study but was indexed by plasma lactate concentrations. Plasma lactate was metabolically cleared by PEG-20k LVR solutions and prevented from rising again, even in the low volume state, presumably because the oxygen debt was being repaid. Furthermore, bioenergetic (lactate conversion) and cardiovascular homeostasis was maintained by these solutions alone, which indicate the animals were induced to tolerate the new low volume state. This is in stark contrast to the saline volume controls where the pigs continued to rapidly accumulate oxygen debt (lactate) and displayed very low cardiovascular function after LVR until they died shortly thereafter. The oxygen repayment after PEG-20k LVR is further evidenced by the return of cardiovascular function and even hyper-dynamic responses seen 1-2 hours after LVR. The increased cardiac output above baseline values represents an increase in oxygen delivery to the periphery, which is consistent with debt repayment. Other data supporting debt repayment include hyperemia in the splanchnic bed and normalization of bicarbonate, lactate, and blood pH. Rapid oxygen debt repayment is indicative of survival after resuscitation (13). An induction of complete metabolic and cardiovascular tolerance to the low volume state in the absence of full resuscitation may be a huge advantage to shock patients in the pre-hospital military and civilian setting where the golden hour needs to be expanded in the absence of whole blood or blood products. Therefore, PEG-20k based crystalloid low volume resuscitation solutions may be useful under those emergent conditions.

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Figure Legends

Figure 1. Comparison of times to complete various tasks in the experimental protocol for both the saline control group and the PEG-20k group. BL, Baseline; HS-0, the start of Hemorrhagic Shock; LVR-0, the start of the Low Volume Resuscitation period; PEG, Polyethylene Glycol-20k. * $P<0.05$ between the two groups, values are mean +/- SD, n=5 per group.

Figure 2. Plasma lactate concentrations in pigs during the time course of the shock and resuscitation protocol (Panel A). PEG, 10% Polyethylene Glycol-20k LVR solution. * $P<0.05$ between corresponding values or compared to the last value recorded in the saline group before death. Panel B shows the LVR times for both groups of pigs. * $P<0.05$ relative to saline for both LVR time and the end lactate values. Values are mean +/- SD, n=5 for each group.

Figure 3. Cardiovascular endpoints in pigs during the shock and resuscitation protocol in pigs given an LVR solution consisting of saline or 10% PEG-20k. A. Mean Arterial Pressure (MAP), B. Cardiac Output (CO), C. Heart Rate (HR). All values are mean +/- SD, n=5 for each group, * $P<0.05$ between corresponding values or to the last value recorded in the saline group before death.

Figure 4. Systemic venous oxygen saturation (SvO_2) measured in the mixed systemic venous blood of the pulmonary artery by the optical catheter during the shock and resuscitation protocol in pigs given a saline or a PEG-20k based LVR solution. Values are mean +/- SD, n=5 for each group, * $P<0.05$ between corresponding values or to the last value recorded in the saline group before death.

Figure 5. Blood flow measured in a branch of the superior mesenteric artery (SMA) during the shock and resuscitation protocol in pigs given an LVR solution consisting of saline or 10% PEG-20k. Values are mean +/_ SD, n=5 for each group, * $P<0.05$ between corresponding values or to the last value recorded in the saline group before death.

Figure 6. Arterial blood gas HCO_3^- (panel A) and pCO_2 (panel B) measured during the shock and resuscitation protocol in pigs given a saline or a 10% PEG-20k based LVR solution. Values are mean +/- SD, n=5 for each group, * $P<0.05$ between corresponding values or to the last value recorded in the saline group before death.

Figure 1

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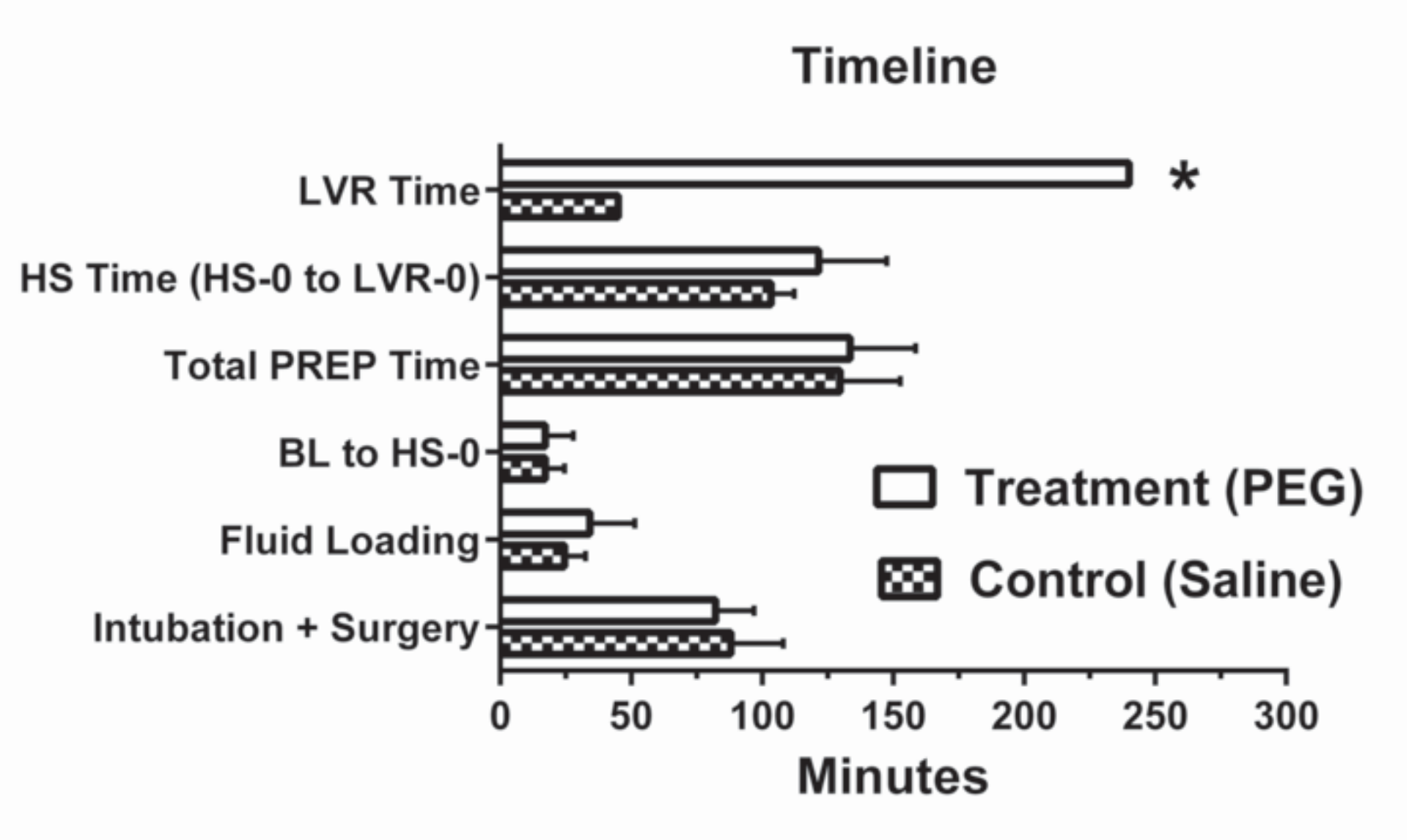


Figure 2

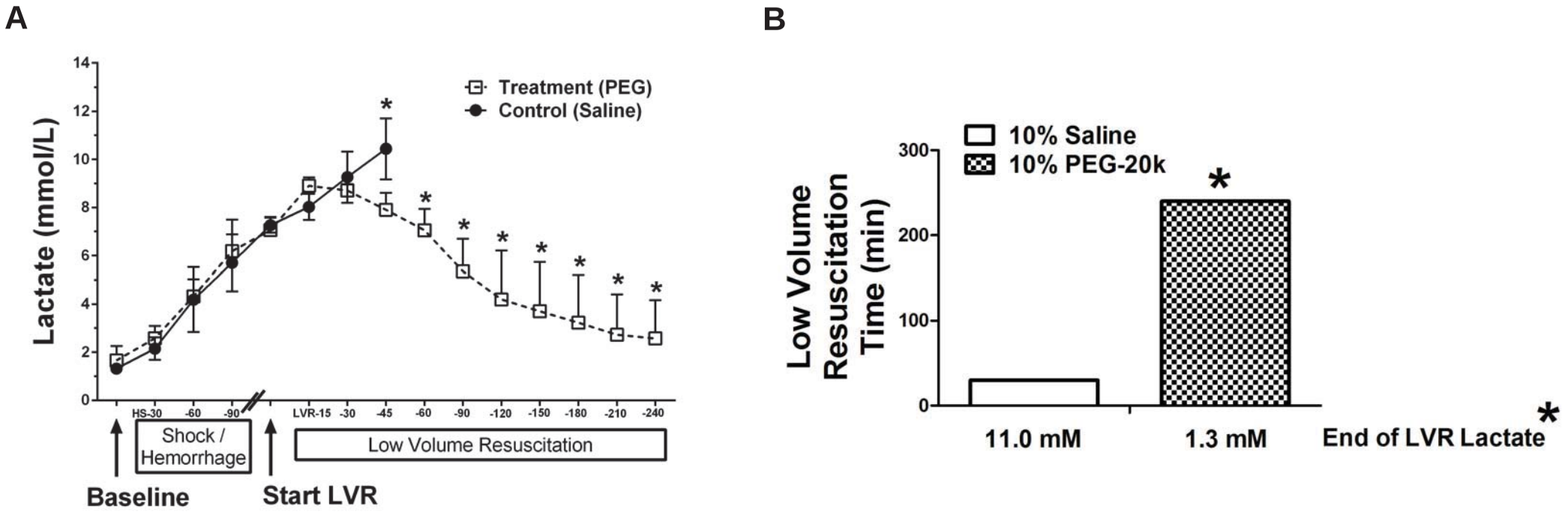
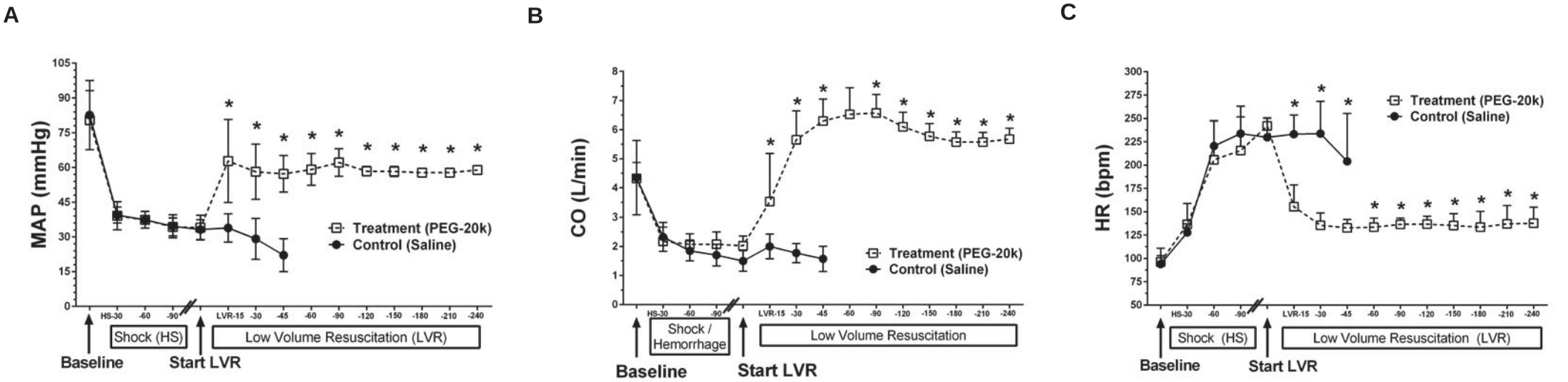

A
Lactate (mmol/L)
Treatment (PEG)
Control (Saline)
HS-30
-60
-90
LVR-15
-30
-45
-60
-90
-120
-150
-180
-210
-240
Shock / Hemorrhage
Low Volume Resuscitation
Baseline
Start LVR
B
Low Volume Resuscitation Time (min)
10% Saline
10% PEG-20k
11.0 mM
1.3 mM
End of LVR Lactate

Figure 3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MAP (mmHg)
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LVR-15
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-60
-90
-120
-150
-180
-210
-240
Treatment (PEG-20k)
Control (Saline)
Shock (HS)
Low Volume Resuscitation (LVR)
Baseline
Start LVR
B
CO (L/min)
8
7
6
5
4
3
2
1
0
Treatment (PEG-20k)
Control (Saline)
Shock / Hemorrhage
Low Volume Resuscitation
Baseline
Start LVR
C
HR (bpm)
300
275
250
225
200
175
150
125
100
75
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Treatment (PEG-20k)
Control (Saline)
Shock (HS)
Low Volume Resuscitation (LVR)
Baseline
Start LVR

Figure 4

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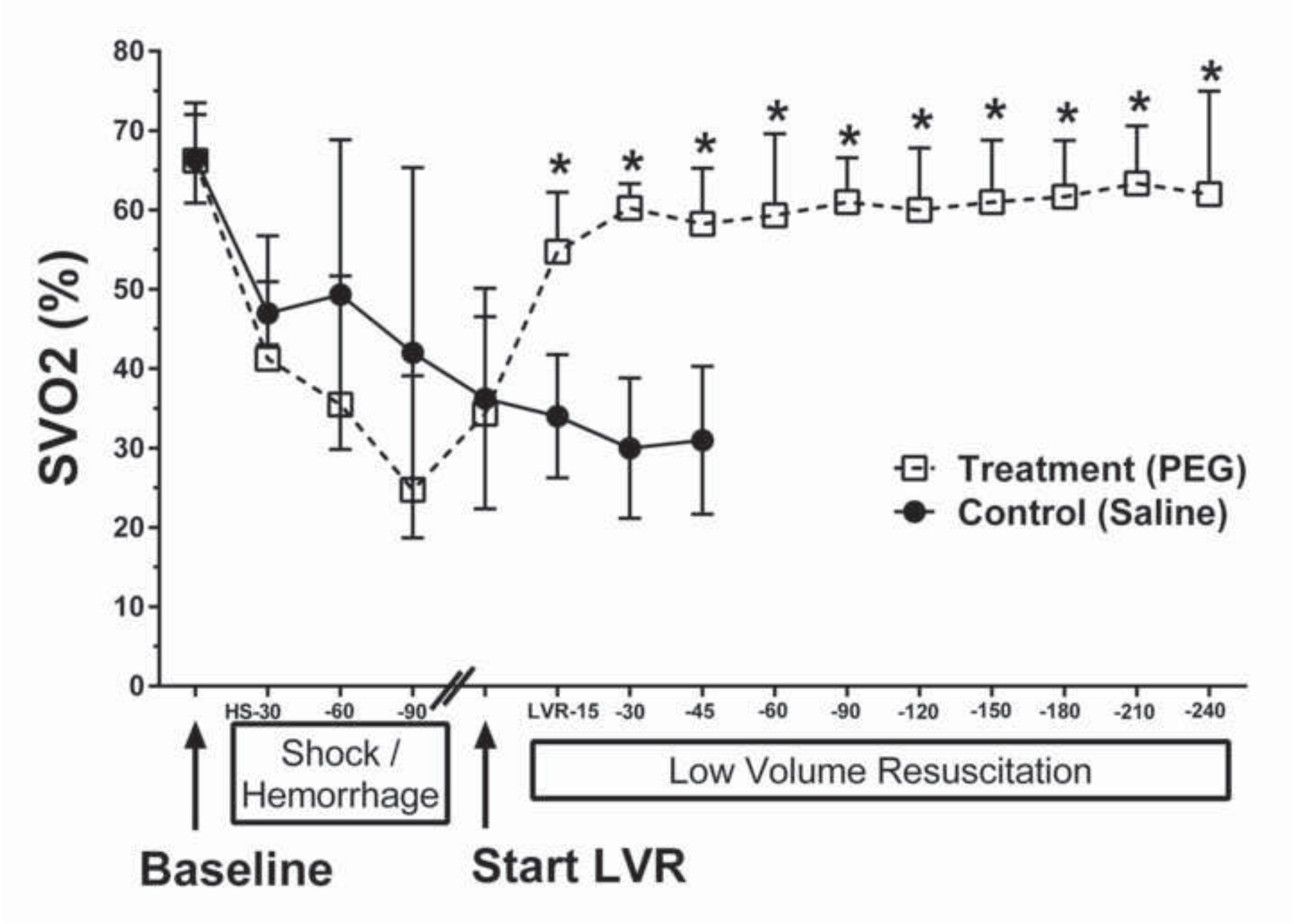

Figure 5

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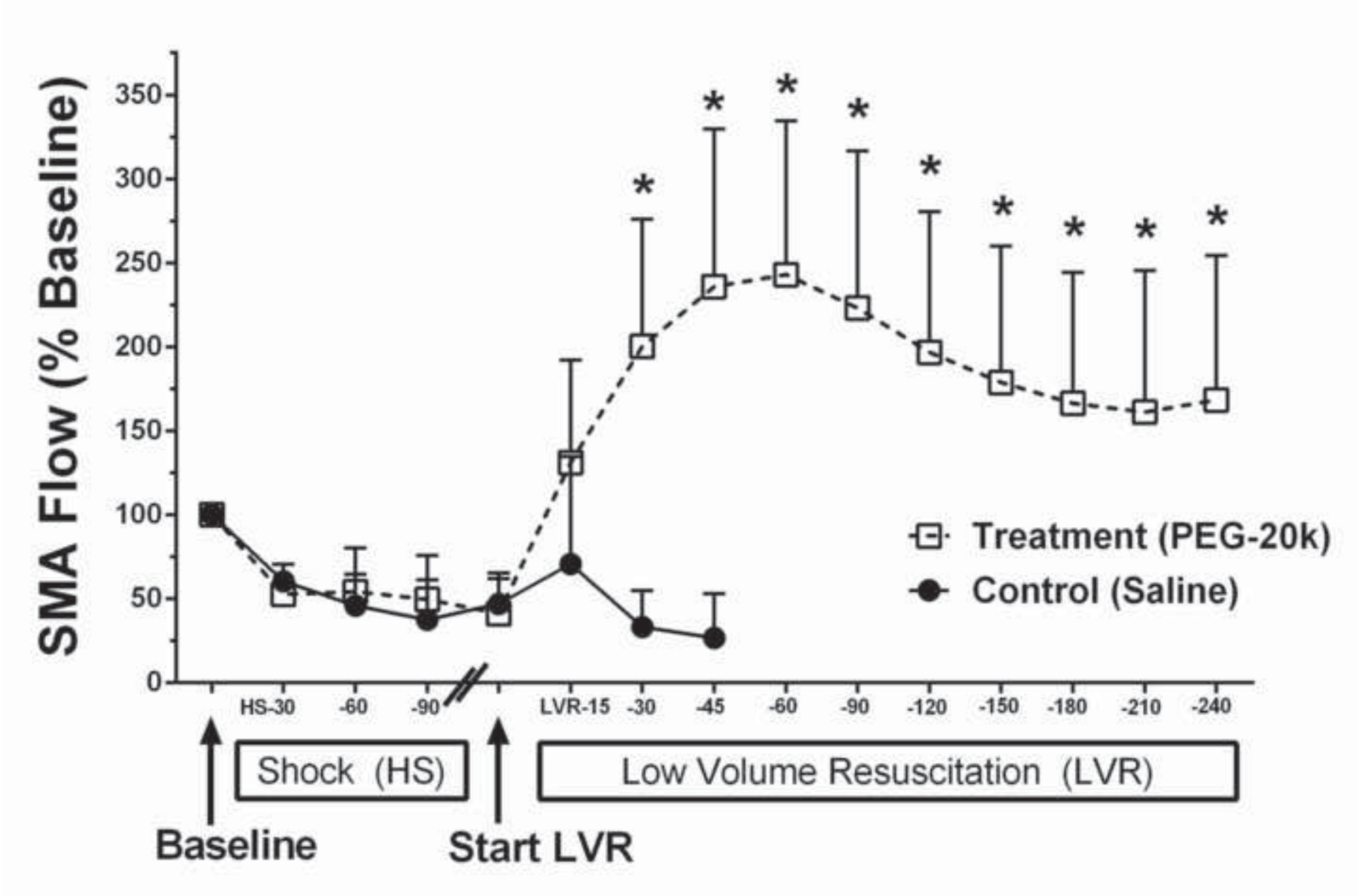
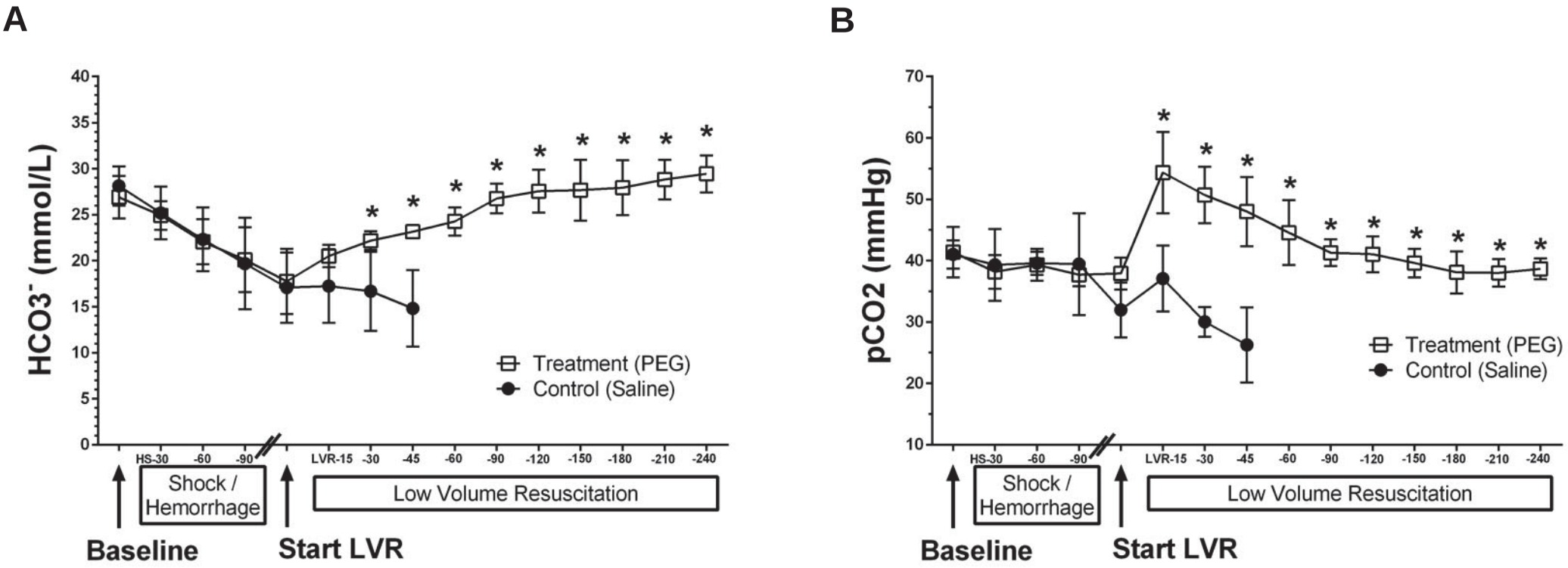


Figure 6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HCO3- (mmol/L)
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HS-30
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LVR-15
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Treatment (PEG)
Control (Saline)
Shock / Hemorrhage
Low Volume Resuscitation
Baseline
Start LVR
B
pCO2 (mmHg)
70
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50
40
30
20
10
Treatment (PEG)
Control (Saline)
Shock / Hemorrhage
Low Volume Resuscitation
Baseline
Start LVR